Bioaccumulation and Sub-lethal Effects of Intermittent and Continuous Exposure to Metals on the Marine Mussel, *Mytilus galloprovincialis*

By

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Abstract

Aquatic organisms experience intermittent exposure to contaminants. The hazard from such discontinuous exposure may not simply be predicted from existing continuous exposure. Little is known about the bioaccumulation responses of shellfish to metals during intermittent compared to the continuous exposure counterparts. This study was designed to compare the bioaccumulation responses of two metals (Cd and Hg) singly (Chapters 3 and 4) or mixtures (Chapters 5 and 6) during continuous and intermittent exposure. Responses were examined using a multi-assay approach in areas of oxidative stress, osmoregulation, haematology and organ pathology. Tissue metal concentrations were highest in the gill and digestive gland for both metals. The linear or curvilinear pattern of accumulation was observed in the continuous exposure. While an alternating temporal uptake and clearance corresponding with the exposure was observed in the gill (Hg alone) and haemolymph (Cd alone) in intermittent profile. At the end, the study shows that accumulation of Hg or Cd was less in the intermittent compared to the continuous exposure for most tissue. Sub-lethal responses during Hg or Cd alone were similar in both regime apart from the severe pathology in the gill (continuous exposure) and inflammation in the digestive gland (intermittent exposure) observed in the Hg alone treatment (Chapter 4). For Hg plus Cd mixture, the additive effect in accumulation was observed in both exposures. Sub-lethal responses during mixtures were not affected in the intermittent. However, an antagonistic interaction was observed in the TBARS during continuous exposures for all tissues apart from the digestive gland. In terms of risk assessment, regulations for the single metals in continuous exposure will be protective for intermittent events at least for adult *M.galloprovincialis*.

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Abbreviations

Abbreviations, symbols and acronyms used in the thesis excluding those equations are defined below.

Abbreviation	Glossary
1-CFOK	One-compartment first order kinetics
µg l^{-1}	Microgram per litre
µm	Micrometer
%	Percentage
°C	Degree centigrade
<	Less than
>	Greater than
N/A	Not available
ADME	Absorption, distribution, metabolism, excretion
Al	Aluminium
ANOVA	Analysis of variance
BFC	Bakers formal calcium
Ca^{2}+	Calcium ion
CAUC	Critical area under the curve
CCME	Canadian Council of Ministers of the Environment
Cd	Cadmium
$CdCl_2$	Cadmium chloride
Co	Cobalt
Ct	Connective tissue
CTO	Critical target occupation
Cu	Copper

DAM	Damaged assessment model
DeBtox	Dynamic energy budget toxicology
DOM	Dissolved organic matter
Dt	Digestive tubules
DTNB	5,-5'-dithiobis-(2-nitrobenzoic acid
EC	European Commission
EC_{50}	Median effective concentration
EDTA	Ethylenediamine tetra acetic acid
ERA	Environmental risk assessment
g (RCF)	g-force (relative centrifugal force)
GR	Glutathione reductase
GSH	Reduced glutathione
GSSH	Oxidized glutathione
GSSTNB	Mixed disulphide
Fc	Frontal cilia
h	Hour
H_2O_2	Hydrogen peroxide
H and E	Haematoxylin and Eosin
HEPES	4-(2-hydroxylmethy) piperazine-1-ethane sulfonic acid
Hg	Mercury
$HgCl_2$	Mercuric chloride
Hi	Haemocyte infiltration
Hy	Hyperplasia
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
IUPAC	International Union of Pure and Applied Chemistry

K^+	Potassium ion
KCl	Potassium chloride
Lc	Lateral cilia
LC_{50}	Median lethal concentration
Lfc	Laterofrontal cilia
$LiCO_3$	Lithium Carbonate
Log_{10}	Logarithm to base 10
Ls	Lacuna space
LSD	Least significant difference
M	Molar
MDA	Malonialdehyde
Mg^{2+}	Magnesium ion
MLET	Median lethal exposure time
mmol l^{-1}	Millimole per litre
mosmol Kg^{-1}	Milliosmole per kologram
MSW	Municipal solid waste
MT	Metallothionein
n	Number of test organisms
Na^+	Sodium ion
NaCl	Sodium chloride
NAO	National Audit Office Environment Agency
NaOH	Sodium hydroxide
ND	Not detected
Ni	Nickel
nm	nanometer
nmol g^{-1} ww	Nanomole per gram wet weight

nmol mg^{-1}	Nanomole per milligram protein
NOEC	No observed effect concentrations
NRR	Neutral red retention
OECD	Organisation for Economic Co-operation and Development
OD	Optical density
p	probability
Pb	Lead
PCP	Pentachlorophenol
$peLT_{50}$	Median post exposure lethal time
pH	Power of hydrogen (The negative log of hydrogen ion concentration in a water-based solution: it measures the acidity or alkalinity of a solution).
Pi	Number of points counted on the counting lattice
POP	Persistent organic contaminants
PPT	Parts Per Thousand
P_T	Total number of points on the counting lattice
PULSETOX	Pulse-exposure model
$t_{1/2}$	Biological half life
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TDM	Threshold damage model
TKTD	Toxicokinetics, Toxicodynamic
TNB	5-thionitrobenzoic acid
TWA	Time weight average
r	Pearson's correlation coefficient
r^2	Coefficient of determination

ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SEM	Standard error of the mean
-SH	Sulphydryl
TBARS	Thiobarbituric acid reactive substances
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
THC	Total haemocyte count
$U\ l^{-1}$	Unit per litre
UK	United Kingdom
UNEP	United Nations Environmental Programme
$U\ ml^{-1}$	Unit per milllilitre
Vi	Volume fraction
v/v	Volume/volume
w/v	Weight/volume
WFD	Water Framework Directive
WHO	World Health Organisation
WQC	Water quality criteria
WQS	Water quality standard
Zn	Zinc

Dedication

This work is dedicated to the loving memory of my mentor, the former Head of Department, Fisheries and Aquatic Environment, RSUST, Late Chief Mckenzie Benibo Inko-Tariah, whose dream was for me to have a degree in Aquatic Ecotoxicology. He passed on the day I departed for this degree. Thoughts of him will ever be green and may his gentle soul rest in the bosom of our Lord and Saviour Jesus Christ.

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List of Publication

- Amachree, D., Moody, A. J and Handy, R. D. 2013. Comparison of Intermittent and Continuous Exposures to Cadmium in the Blue Mussel, *Mytilus edulis*: Accumulation and Sub-lethal Physiological Effects. *Ecotoxicology and Environmental Safety Journal*, 95, 19-26.

- Amachree, D., Moody, A. J and Handy, R. D. 2014. Comparison of Intermittent and Continuous Exposures to inorganic mercury in the Mussel, *Mytilus edulis*: Accumulation and Sub-lethal Physiological Effects. *Ecotoxicology and Environmental Safety Journal*, 109, 133-142.

Oral presentations

- Amachree, D. 2011. Comparison of Intermittent and Continuous Exposures to cadmium in Common Mussel, *Mytilus edulis*: Accumulation and sub-lethal effects. Ecotoxicology Research and Innovation Centre, School of Biological Sciences (SoBBS). Plymouth University, February, 2011.

- Amachree, D., Moody, A. J and Handy, R. D. 2012. Comparison of Intermittent and Continuous Exposures to Mercury in the Marine Mussel, *Mytilus edulis*: Accumulation and Sub-lethal Effects. Society of Environmental Toxicology and Chemistry (SETAC) UK, and Royal Society of Chemistry (RSC) joint meeting. "Bioavailability: Linking Complex Environmental Chemistry with Environmental Response" 17-18th September, 2012. The RSC Chemistry Centre, Royal Society of Chemistry, Burlington House, Piccadilly, London, W1J 0BA, UK. Abstract book, No S1.4, p11-12.

- Amachree, D. 2013. Comparison of Intermittent and Continuous exposure to metals in *Mytilus edulis*: Accumulation and sub-lethal physiological effects. School of Biological Sciences (SoBBS) Seminar series, Plymouth University, 26th February 2013.

Poster presentation

- Amachree, D., Moody, A. J and Handy, R. D. 2011. Comparison of Intermittent and Continuous Exposures to Cadmium in Common Mussel, *Mytilus edulis*: Accumulation, Biochemistry, Immunology and other physiological Effects. Society of Environmental Toxicology and Chemistry, SETAC UK annual meeting, "Polluted Planet: Sustaining Ecosystems and Biodiversity" 13th September 2011 Charles Darwin House, London, UK. Poster No. S1.13.

- Amachree, D and Handy, R. D. 2012. Intermittent Vs Continuous Exposure to Cadmium: Tissue Accumulation and Pathology. Postgraduate Society Annual Conference, 2012. "The Achievements of the Past and the Promises of the future" 26th June, 2012. Plymouth University, UK. Poster.

- Amachree, D., Moody, A. J., and Handy R. D. 2013. Comparison of Intermittent and Continuous Exposures to Inorganic Mercury in the mussel, *Mytilus edulis*: Accumulation and Sub-lethal Physiological Effects. Society of Environmental Toxicology and Chemistry, SETAC Europe 23rd annual meeting, "Building a better future: Responsible Innovation and Environmental Protection", 12-16th May 2013, Glasgow, U.K. Scottish Exhibition plus Conference Centre (SECC), Glasgow, UK. Abstract book No. TU012, p218.

- Amachree, D., Moody, A. J., and Handy R. D. 2013. Intermittent exposure to a mixture of mercury and cadmium in the blue mussel, *Mytilus edulis*: interactive effect on metal accumulation. Joint Annual Meeting of the Ecotoxicology Research and Innovation Centre Plymouth University and the Society of Environmental toxicology and Chemistry UK Branch. ''Chemistry at the Biological Interface: From Environmental Monitoring to Molecular Biology''. Plymouth Lecture Theatre, Portland Square Building, 9-10th September, 2013, Plymouth University, UK. Poster No. ERIC. P12.

Workshops Attended

- Risk assessment training workshop. Society of Environmental Toxicology and Chemistry (SETAC)-UK, 2012. Charles Darwin House, London, UK.

- Practical techniques in molecular biology workshop. Postgraduate Society 2012, Plymouth University, UK.

- How to best conduct aquatic ecotoxicity tests according to the International Guidelines. Society of Environmental Toxicology and Chemistry (SETAC) - Europe Annual meeting 2013. Scottish Exhibition plus Conference Centre (SECC), Glasgow, UK.

- Scientific writing skills for environmental scientists. Joint ERIC/ SETAC-UK training event 2013. Babbage building, Plymouth University, 8th September, 2013.

Academic courses attended with dates

Date	Academic courses
Sep-Nov 2010	ENV 5101 Demonstration (80%)
Sep-Oct 2013	Graduate Teachers Associates (GTA)

Skilled courses attended with dates

Date	Skilled course
28-04-2010	Giving and receiving feedbacks
06-05-2010	Research-owning and using
10-05-2010	Preparing effective poster presentations
11-05-2010	Overview to searching and accessing information resources
19-05-2010	Quantitative research
27-05-2010	Word master document
28-05-2010	Word: Proofing and tracking changes
07-06-2010	MS Project
09-06-2010	Introduction to Endnote
10-11-2010	Presenting to an audience part 1
19-11-2010	Stress management
22-11-2010	Transfer process
11-03-2011	Powerpoint presentation 2001: enhancing your presentation
04-05-2011	Developing Professional writing skills
26-05-2011	Introduction to Pebblepad
27-05-2011	SPSS
02-11-2011	SPSS
18-11-2011	Making progress: avoiding defeatism and self-sabotage

14-03-2012	Originality and plagiarism
27-03-2012	An introduction to applying for research funding
28-03-2012	Leadership and management: selection interviewing
5-11-2012	Effective reading
5-11-2012	Preparing for viva
8-11-2012	Getting the most from public engagement
26-11-2012	Media training: getting your research into the media
30-01-2013	Excel 2012: using Excel as a database
01-05-2013	Writing up and completing the thesis
29-10-2013	Keeping laboratory records

Conference attended with dates

Date	Conference	Conference title	Status
17- 06-2010	PG Society Short Conference	-	Audience Member
2010	International Student Conference	Unlock your employment	Audience Member
20-12-2010	Marine Institute conference	Spirit of discovery: Plymouth diverse marine and maritime research	Audience member
10-02-2011	Ecotoxicology Research and Innovation Centre, Research group meeting	-	Oral Presentation
17-03-2011	PG Society Short Conference	-	Audience Member
04-04-2011	Ecotoxicology Research and Innovation Centre, 1st Annual Conference	Environmental contamination: Chemical and Biological Approaches for Protecting Organisms	Audience member
13-09-2011	Society of Environmental Toxicology And Chemistry (SETAC)-UK Annual meeting 2011	Polluted planet: sustaining systems and biodiversity	Poster presentation
14-09-2011	Society of Environmental Toxicology And Chemistry (SETAC)-UK workshop	Risk assessment training workshop	Trainee
2010, 2011	School of Biomedical and Biological Sciences seminar series	-	Audience member
27-05-2011	International Student Conference	Next steps to further success	Audience Member
29-06-2011	PG Society Short Conference	-	Audience Member

21-10-2011	Ecology and Marine Biology seminar series 2011	Sex changes in crustaceans:contamination, parasitism or both?	Audience Member
23-11-2011	PG Society Short Conference	-	Audience Member
14-03-2012	PG Society Short Conference	-	Audience Member
26-06-2012	PG Society Annual Conference	The achievements of the past and the promises of the future	Poster presentation
13-06-2012	Ecotoxicology Research and Innovation Centre, 2nd Annual Conference	Building International Collaborations in Environmental Toxicology and Chemistry	Poster presentation
16/19-07-2012	Postgraduate Society workshop	Practical techniques in molecular workshop	Trainee
17/18-09-2012	Society of Environmental Toxicology And Chemistry (SETAC)-UK Annual meeting 2012	Bioavailability:Linking Complex Environmental Chemistry with Environmental Response	Oral presentation
06-02-2013	School of Biomedical and Biological Sciences	Seminar series	Oral presentation
12/16-05-2013	Society of Environmental Toxicology And Chemistry (SETAC)-Europe 23rd Annual meeting 2013	Building a better future:responsible innovation and environmental protection	Poster presentation
18-06-2013	PG Society Annual Conference		Audience
9/10-09-2013	ERIC and SETAC-UK Joint Annual meeting	Chemistry at the biological interface:from environmental monitoring to molecular biology	Poster presentation

Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author has been registered for any University award without the prior agreement of the Graduate Committee.

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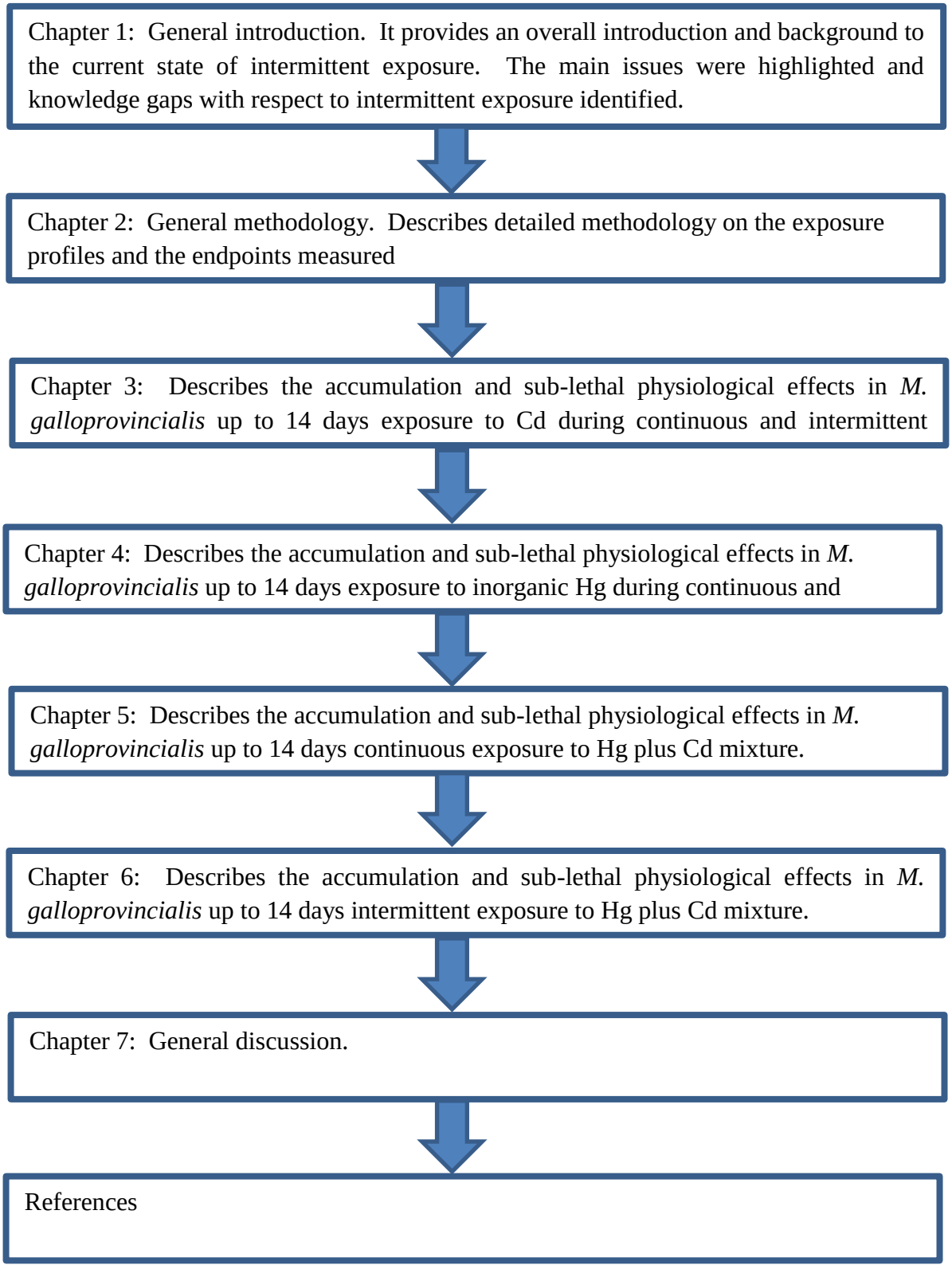
Thesis outline



Chapter 1 ***:***

General Introduction

1.0. Intermittent Contamination

The quality of many receiving waters, especially in the developed countries has improved since the implementation of regulations to reduce direct discharges in the 80s (McCahon and Pascoe, 1990). However, as these regulations reduce contamination from the point sources, the impact from diffuse sources becomes evident (NAO, 2010). For example, in 2010 the National Audit Office (NAO) reported that intermittent and transient contamination from diffuse sources (e.g. farming, land runoffs or wrongly connected domestic or commercial drainage systems) are the major source of poor water quality in England and a significant reason water bodies not currently be meeting Europeans standards (NAO, 2010).

Intermittent exposure over the years has been considered as an ideal and more environmentally realistic mode of contamination (Handy, 1994; Hickie et al., 1995). Intermittent (also termed: episodic, pulse, sporadic) contamination involves one or more brief episodes of contamination where the concentration of the contaminant(s) varies over time to give a dynamic rather than steady-state exposure (Handy, 1994; Hickie et al., 1995; Reinert et al., 2002). Accounting for the effects from intermittent contamination event for environmental risk assessment is a challenge (Ashauer and Brown, 2013). Particularly, given that in such contamination event, the highest (peak) concentration may only be present for a short period of time and might greatly exceed the background concentration (Handy 1994; Liess et al., 1999). Also, in situations where the temporal peak concentration exceeds the toxicological threshold (especially for highly bioaccumulative chemicals) delerious effects may be observed during or after the event causing a delayed or latent effect on organisms (Zhao and Newman, 2004; Alonso and Camargo, 2009).

In spite of the difficulties in assessing the effects from intermittent contamination, water quality criteria for intermittent events has been derived from the

no observed effect concentration (NOEC, the highest test concentration with a mean response not statistically significantly different from that of the control) obtained from standard ecotoxicity tests. The assumption is that at an equivalent dose, the response of organisms is equal to those in the continuous exposure (at least for pesticides, see review, Boxall et al., 2002). There are concerns that the NOEC derived from the standard toxicity test may not be protective for an intermittent contamination event (Handy, 1994; Hickie et al., 1995; Diamond et al., 2006).

Standardised regulatory toxicity tests are usually designed to evaluate the effects of continuous exposure to a fixed concentrations of a chemical substance to organisms under stable (steady-state) environmental conditions, and then toxicological parameters (e.g., LC_{50}, median lethal concentration, EC_{50}, median effective concentration), or other responses (mortality, immobility, alteration of feeding activity, etc.,) are calculated over a specific time (Pascoe and Shazili, 1986; McCahon and Pascoe, 1990; Hickie et al., 1995; Crompton, 1997; Cold and Forbes, 2004). The rationale for the endpoint used in such experiments is that the biological response is normally associated with the exposure concentration of the chemical in a predictable way (the dose-response relationship) so that there is confidence in setting water quality objectives/regulations that are based for example on the NOEC from the experiments (Crane and Newman, 2000). Unfortunately, the protocol for standard test does not reflect real environmental scenarios, since contamination (with exemptions to persistent organic contaminants, POPs) in nature is unpredicted, short-lived and the concentrations of chemicals fluctuate with time (Handy, 1994).

The ecotoxicological concern is that short term repeated intermittent exposures may be more toxic when compared to the continuous counterpart, or long contamination event of equivalent doses (Pascoe and Shazili, 1986; Handy, 1995): such that in risk assessment, the NOEC values from the standard test may either overestimate (if

expected environmental concentrations are never reached or if the exposed organisms have the ability to recover the toxicity for an intermittent contamination (Handy, 1995). Alternatively, it may underestimate toxicity if the brief exposures can cause adverse effects (Cold and Forbes, 2004; Traas et al., 2004; Alonso and Camargo, 2009). According to Handy (1994) the overall accumulation response to intermittent contamination events depend mainly on the duration of peak concentrations and any recovery periods between multiple episodes relative to the contaminants uptake and depuration rates of the organism. Unfortunately, the protocol used in the standard toxicity tests does not make an assessment of the toxicity of intermittent exposure (Ashauer et al., 2007b). This standardization and simplicity in regulatory toxicity testing are a weak link in extrapolating result from such tests to real natural contamination events (Luoma, 1995). Therefore, one should not apply (or heavily modify) steady-state concepts used for protecting the environment to assess intermittent contamination.

1.0.1. Sources of Intermittent Contamination

Intermittent contamination can arise from various sources which include periodic releases during industrial processes (e.g., weekly production cycle in a factory) or applications of agrochemicals which are often cyclic or repetitive. Accidental oil spillage, which are by nature isolated and unpredicted events (e.g., the recent 2010 Gulf of Mexico oil spill), waste inputs from farm, atmospheric deposition, urban and domestic activities that produce run-off depending on the local rainfall pattern. Also included are trade and sewerage effluents from production cycle of local industry that vary in quantity and chemical composition (Oikari et al., 1985; McCahon and Pascoe, 1990; Handy, 1994; Martinovic et al., 2008).

Natural biogeochemical processing can also create variability in exposure profiles. For example, environmental contamination may be removed from the water column by natural processes such as aggregation, sorption or complexation with colloids (Lead and Wilkinson, 2006). The concentration of contaminants themselves may change by simple dilution in the receiving waters, photo/ bio-gradation, bioremediation or adsorption into sediments. Migration of organisms from sites of contamination to clean areas can cause relative spatial variations in the exposure profile (Luoma and Rainbow, 2010). A decline in contaminant concentration to below detectable levels for a while might also create variability in the exposure profile. Organisms might have time to eliminate or detoxify the accumulated contaminant and recover from the exposure; especially if the detoxification mechanisms of the organisms are still effective (Mancini, 1983; Pascoe and Shazili, 1986; Ashauer and Brown, 2008). However, if the concentration of the intermittent event exceeds the toxicological threshold, deleterious effects may be observed during or after the event (e.g., for highly bioaccumulative substances where the biological half-life for excretion is long compared to the recovery period in clean water, Handy 1994), as well as causing a delayed biological response (Alonso and Camargo, 2009). Delayed response can also result from redistribution of contaminates from less critical organs to vital organs (Handy, 2010).

1.1. Effects during Intermittent Exposure

Studies on intermittent contamination are relatively few and limited to a few test species and chemicals. For example, reports on intermittent exposure to metals includes: with fishes: Pascoe and Shazili, 1986; Handy, 1992; Chen et al., 2012; and invertebrates: Coleman, 1980; Viarengo et al., 1999; Shuhaimi- Othman and Pascoe,

2007; Amachree et al., 2013. These studies mainly focus on tissue metal accumulation with only limited information on biological effects of the exposure. Other toxicity studies with fish, insects, crustaceans and microsomes showed that responses during intermittent exposures depends on the contaminant concentration (Thurston et al., 1981; Seim et al., 1984; Camargo, 1996), exposure duration (Brown et al., 1969; Pascoe and Shazili, 1986), recovery time (Handy, 1994; Kallander et al., 1997; Traas et al., 2004) and frequency of exposure (Handy, 1994, Naddy and Klaine, 2001); as well as factors normally considered in aquatic toxicological test (e.g., water quality, age of species, nutritional state).

Sub-lethal responses during intermittent exposures can occur in minutes to hours and up to months (Table 1): and can be either less or more in the intermittent exposure compared to the continuous exposures on a range of endpoints. Till date no clear correlation has been found between tissue accumulation and toxicity, suggesting that reliable toxicity indicators for intermittent contamination still remain to be established. For example a recent study from our laboratory mussels showed that Cd accumulation by *Mytilus edulis*, was generally greater during continuous exposure than in an intermittent profile; but despite less Cd accumulation in the latter, the intermittent event was just as toxic in terms of sub-lethal biological responses (Amachree et al., 2013).

Table 1: Some examples of sub-lethal effects of intermittent exposures

Test organisms	Chemicals/ Lab or Field/ salinity or hardness	Concentrations/ duration	Duration of Exposure: Recovery time in clean water	Frequency of exposure	Sub-lethal responses/ life stage	References
Freshwater amphipod (*Eulimnogammarus toletanus*)	Nitrite/lab/ (hardness not reported)	0.5,5.0, 10.0 mg l^{-1} (nominal) /96 h	1h:95h, 8h:88h, 24h:72h, 48h:48h	4	Increased delayed mortality in the post exposure period as compared to the pulsed period	(Alonso and Camargo, 2009)
Freshwater amphipod (*Gammarus pulex*)	Esfenvalerate/ lab (hardness not reported)	0.1-0.6 µg l^{-1} (nominal)/ 2 weeks	I h:2 weeks	1	Significant effect on survival and reproduction in the post exposure period	(Cold and Forbes, 2004)
Rainbow trout (*Oncorhynchus mykiss*) Goldfish (*Carassius auratus*) Fathead minnow (*Pimephales promelas*)	Mercuric Chloride/lab (hardness not reported)	3 µg l^{-1}/120h (nominal)	24h:24h	3	Tissue accumulation is less in the intermittent as compared to the continuous exposure regime. No effects in target organs or the distribution of the body burden, lower tissue concentration	(Handy, 1995)
Fresh water microcosm	Chlorpyrifos/lab (hardness not reported)	0.01-35 µg l^{-1} (nominal)	63 d:6 months	1	Little or no recovery from the effects	(Traas et al., 2004)

Continuation of Table 1: Some examples of sub-lethal effects of intermittent exposures

Test organisms	Chemicals/lab or field salinity or hardness	Concentrations/ duration	Duration of exposure: Recovery time in clean water	Frequency of exposure	Sub-lethal responses/ life stage	References
Mussel, *Mytilus edulis*	Copper: Cadmium (salinity 6.3-6.5 ppt)	0.1-5.0 mg l^{-1}: 0.5-25 mg l^{-1} (nominal)	24h:21 d	1	Decreased rate of valve opening. Increased mortality. Decreased byssogenesis. Increased mucus secretion in the gills/ juvenile and adult mussels	(Sunila, 1981)
Mussel, *Mytilus galloprovincialis*	Cadmium (Lab and field) (salinity 35 ppt)	200 µg l^{-1} (nominal)	7 days in aquaria followed by 28 days detoxification in sea		Induction of MT. Mussel later exposed to 300 µg l^{-1} Fe caused a Cd-dependent resistance to oxidative stress/ adult mussels	(Viarengo et al., 1999)
Tilapia, *Oroechromis mossambicus*	Copper (lab) (hardness not reported)	30-90 µg l^{-1} larvae and juvenile; 100-300 µg l^{-1} adult/10 d (nominal)		2	Substantial effect on survival rates/ larvae, juvenile and adult life stages	(Chen et al., 2012)
Seagrass, *Zostera capricorni*	Copper;Irgarol 1051 (field) (salinity not reported)	5 mg l^{-1} Cu;100 µg l^{-1} Irgarol/ (nominal)	10 h:4 d	2	Effects on photosynthesis	(Macinnis-Ng and Ralph, 2004)

Continuation of Table1: Some examples of sub-lethal effects of intermittent exposures

Test organisms	Chemicals/ lab or field. Salinity or hardness	Concentrations/ duration	Duration of exposure: Recovery time in clean water	Frequency	Sub-lethal responses/life stage	References
Mussel, *Mytilus edulis*	Cd (Lab) (Salinity, 34.0 ± 0.1 ppt)	56 µg l^{-1} Cd as $CdCl_2$ (measured)	2 d: 2d. a total of 14d	4	Tissue accumulation less in the intermittent compared to the continuous exposure. Biological responses were either same or less in the intermittent compared to the continuous/ adult mussels	(Amachree et al., 2013)
Mussel, *Mytilus edulis*	Hg (Lab) (Salinity 35.5 ± 0.1 ppt)	48 µg Hg l^{-1} as $HgCl_2$ (measured)	2 d: 2d. a total of 14d	4	Tissue accumulation less or equal in the intermittent compared to the continuous exposure. Digestive gland in the intermittent exposure showed severe inflammation compared to the continuous exposure/ adult mussels	(Amachree et al., 2014)
Marine mussel *(Mytilus edulis)*	Cadmium/ Lab (salinity 30-33 ppt)	0.035-0.35 mg l^{-1} (nominal)	15 d: > 9h daily	13	Tissue accumulation in the intermittent exposure is less than the continuous. Accumulation is < 2 fold but >1 in the intermittent regime/ adult mussel	(Coleman, 1980)
Daphnia magna	Dimethoate; Pirimicarb	10-30 mg l^{-1} (Dimethoate) or 40-100 µg l^{-1} (pirimicarb) (nominal)	0.5-8h: 21d (dimethoate) or (pirimicarb)	1	Both pesticides caused reproductive damage in <4h. Increased mortality in animals that apparently had recovered after a single pulse/ 24h old.	(Andersen et al., 2006)

1.2. Predicting Toxicity of Intermittent Exposure

Due to the complexity of measuring toxicity of an intermittent event, several attempts have been made to measure directly or develop models to assess the toxicity of the intermittent contamination event. Direct experimentation that identifies the importance of magnitude, duration, frequency of exposure and recovery periods during intermittent event have been reported (Wright, 1976; Green et al., 1988; Kallander et al., 1997). To date different approaches have been used to develop toxicokinetic and toxicodynamic (TKTD) models in an attempt to assess the toxicity of the intermittent event. For example Abel and Garner (1986) proposed the medium lethal exposure time (MLET) as an end point, which is the time required to kill half of the organism within a set post–exposure period. Pascoe and Shazili (1986) proposed the median post exposure lethal time ($peLT_{50}$) which is the time from the end of the exposure period when 50% of the organism are killed. Mancini (1983) developed a kinetic uptake-depuration model based on the assumption that the concentration of a chemical in an organism at any one time is a function of the uptake and detoxification rates. Hickie et al., (1995) pulse-exposure model (PULSETOX) was based on the simple one-compartment first order kinetics (1-CFOK) equation. Ashauer et al., (2007a) developed the threshold damage model (TDM) and a method based on time-weighted average concentrations (TWA pulses), both models link fluctuating exposure to effects. Also, the dynamic energy budget toxicology (DEBtox), the damaged assessment model (DAM), the critical target occupation (CTO) and the critical area under the curve (CAUC) models were developed (see Ashauer et al., 2006, Ashauer and Brown, 2008 for review of models). These mathematical models have proved reasonably accurate in predicting toxicity of single contaminants (e.g., zinc, Mancini, 1983; pentachlorophenol (PCP), Hickie et al., 1995), or mixtures as well as addressing the challenges of assessing toxic effects resulting from pulsed exposure to toxicant (Ashauer and Brown, 2013).

The success of the models depend on how representative the parameters are of the exposure pattern such as length, number, duration of peaks, intervals between peaks (Ashauer et al., 2013). Up till date there have not been a consensus on which model and approaches are most suitable for sub-lethal endpoints as well as the harmonisation of TKTD modelling (Ashauer and Brown, 2013).

1.3. Metal Contamination in the Aquatic Environment

Metal contamination in aquatic environment is one of the most ubiquitous and persistent environmental issues (Luoma and Rainbow, 2005). Metals are natural components of the biosphere (Bryan, 1971; Luoma, 1983). Metal contamination in coastal waters are through natural and anthropogenic inputs from rivers, urban storm water runoff, effluents from industries, sewage, mining and smelting operations (UNEP, 1983; Connell and Miller, 1984). Although some metals such as zinc, copper and cobalt are absolutely essential for normal growth and development for living organisms (Bryan, 1971; Engel and Sunda, 1979; Luoma, 1983; Kamo and Nagai, 2008), others like cadmium (chapter 3) and mercury (chapter 4) are not. At sufficiently high concentrations all metals are toxic to aquatic organisms (Luoma and Rainbow, (2010). For some (e.g., selenium) there is a narrow range of concentrations between what is essential and what is toxic (Engel and Sunda, 1979; Kamo and Nagai, 2008; Nadella et al., 2009).

Metals commonly enter the marine environment as components of freshwater discharges. Metals contained in such discharges are usually sorbed to dissolved ligands and particulate matter, which reduces metal availability to aquatic organisms (Luoma, 1983, Buffle and Leppard, 1995). However, the high dilution ratios that accompany the freshwater discharges favour subsequent metals desorption. Desorption can also be

enhanced in seawater due to the presence of soluble anions (e.g., chloride, sulphate and bicarbonate), which in turn compete for the sorption of metals to form soluble complexes. Competitive exchange of inorganic cations in seawater (e.g., sodium, potassium, calcium and magnesium) on the sorptive substrate may also enhance desorption of metals (Knezovich, 1994).

1.3.1. Mode of Metal Exposure

Exposure of organisms to metals can occur in both dissolved (solution) and particulate phases (food, colloids). Within each phase the organism is exposed to a variety of physiochemical forms of each metals (free ions or complexed forms), and each form may differ in its accessibility (Luoma, 1983; Drexler et al., 2003; Gueguen and Dominik, 2003). Uptake from the particulate phase may occur following ingestion of particles or in some cases by endocytosis of the particles at the body surface (Moore, 2006; Handy et al., 2008). Direct uptake from water occurs by either adsorption onto cell membrane surface or organism surfaces, or absorption across cell walls or body surfaces, or intracellular trafficking and/or storage in membrane–bound compartments (Drexler et al., 2003; Handy and Eddy, 2004). The quantity of metal transported into biological tissues may be influenced by the physiological state of the organism or by the physiochemistry (pH, dissolved organic matter (DOM), presence of other metal ions, temperature, dissolved oxygen, hardness) of the external medium (Luoma, 1983). Uptake is both metal and species-specific (Luoma and Rainbow, 2005).

1.3.2. Metal Accumulation and Toxicity

The fate of metal in an organism depends on processing including absorption, distribution, metabolism and excretion (ADME). The first step in the uptake is the adsorption of the metal to the surface of the organism (Handy and Eddy, 2004) followed by a slower rate of metal accumulation into the body compartments (e.g. tissue or organelles) (Drexler et al., 2003). Organisms with injury tend to take up contaminants more rapidly due to damaged epithelial barriers (e.g., gills, skin, gut), thus enabling uncontrolled diffusion rather than regulated uptake through ion transporters (Handy and Eddy, 2004). In all cases, bioaccumulation will only occur when the net rate of uptake exceeds that of detoxification and/or elimination by the organism (Rainbow, 2002; Drexler et al., 2003). Once absorbed by the organism, metals can bind to biomolecules that are essential to cellular function, alter their function and cause toxicity (Rainbow, 2002; Marsden and Rainbow, 2004). For example, metals such as Hg and Cd have high affinity for -SH (Sulphydryl) group on amino acids such as cysteine, they can form complexes with the -SH groups and change the tertiary structure of cellular protein. Metals can also affect ion transporters. Also, the competitive inhibition of the Ca^{2+} pump by Cd has been demonstrated in freshwater fish (Verbost et al., 1989). Metals may also be precipitated in phosphate or sulphide bodies within cells, thereby sequestering them and preventing the mobility and subsequent toxicity (Drexler et al., 2003).

Different organism show different patterns of accumulation. The precise pattern of accumulation in the body compartment depends on the affinity constants for the ion transporters in each tissue, the number of metal-binding ligands in the tissue, and the blood flow (Handy and Eddy, 2004). However, for molluscs the processes of metal accumulation have not been shown to be different between different groups: though cellular uptake and excretion may be partially affected by the different physiochemical

properties of the medium. In all cases, metal toxicity can only occur when the rate of uptake from all routes (aqueous or dietary) exceeds the combined rate of detoxification and elimination (if present) of the metal concerned. Toxicity does not depend on the total accumulated metal concentration but on the threshold concentration of internal metabolically available metals (Rainbow, 2002; Marsden and Rainbow, 2004).

1.3.3. Metal Uptake and Toxicity in Mussels

Aquatic molluscs take up and accumulate trace metals whether essential or non-essential from both dissolved and particulate phases (Marigómez et al., 2002). Uptake from the dissolved phase is mainly through the gills and to a lesser extent the mantle and the digestive gland being negligible (Cd, *M. edulis*, Scholz, 1980 cited in Marigómez et al., 2002). Once in the gills, metals are bound to metallothioneins (MT), incorporated into the lysosomes, and released basally towards the haemolymph plasma and circulating haemocytes (Marigómez et al., 2002) from where the metals are distributed to the other organs (Roesijadi and Hall, 1981). The major site of uptake from the particulate phase is the digestive gland. After which metals are first transferred to the lysosome, then to the digestive cells of the digestive gland (Marigómez et al., 2002).

Cellular uptake of metals across membranes can occur by passive diffusion (Carpene and George, 1981; Williams, 1981; Marigómez et al., 2002), by facilitated diffusion (Carpene and George, 1981), or by energy-dependent processes (Rainbow and Dallinger, 1993). However, it is believed that marine molluscs do not need energy mediated transport for the free metal ions or cations to cross the cell membrane (Marigómez et al., 2002). Cellular metal uptake can also be enhanced by the synthesis of metal-binding protein (e.g., metallothionein, MT) or by increased formation of

mineralized granules. Specific Cd- (George et al. 1981) and Hg- (Roesijadi and Hall, 1981) binding proteins have been found in Mussels, *M.edulis*. The synthesis of metal binding proteins (e.g., metallothioneins, Hg-binding proteins) during metal exposure have been demonstrated in the accumulation of Hg (gill, Roesijadi, 1982) and Cd (digestive gland, Viarengo et al., 1987) in the *Mytilus* species.

Once in the cell, metals bind to a variety of ligands that maintain an inwardly directed diffusion gradient and prevent efflux of the metal. The rates of intracellular accumulation of metals are determined by the number and binding characteristics of the available ligands and their accessibility (Marigómez et al., 2002). Just as the ligand pool differs from cell to cell, different metals may be retained in different cell types. For example, class ''a'' metals such as sodium, calcium, magnesium are oxygen seeking and are localised in cell types (e.g., connective tissue calcium cells, basophilic cells) rich in carbonate, phosphate, oxalae and sulphate. Whereas the class ''b'' metals such as Cd and Hg are sulphur and nitrogen seeking and as such localised in cells (e.g., gill epithelial cells, haemocytes, digestive cells) rich in sulphur and nitrogen (Nieboer and Richardson, 1980). A classic example is the binding of Hg and Cd to thiol groups of the cysteine residue of metallothioneins, glutathione etc (Rabestein et al., 1985). In *M. edulis*, Cd has been reported to be always associated with sulphur (attributed to the presence of cysteine residue in a metal-binding protein) and frequently with phosphate in the membrane-bound vesicles of the cells of the gill epithelium and haemocytes (Marshall and Talbot, 1979). However, there has been no clear evidence of intranuclear accumulation of metals in molluscs (Marigómez et al., 2002).

Metal toxicity in mussels has been extensively studied (Table 2). Mussels have been shown to avoid contaminants by closing of the valves (Mohan et al., 1987). Mohan et al. (1987) identified the valve closing mechanism after exposing *M.edulis* to organophosphate pesticide (Mohan et al, 1987). Mussels have also been shown to

accumulate high tissue concentrations of contaminants when exposed to sub-lethal concentrations. Alterations in the defence mechanisms, induction of the metal-binding proteins and histological alterations in tissue structure has been proven as toxic effect associated with metals such as Hg, Cd, Cu (Coles et al., 1995; Viarengo et al., 1999; Matozzo et al., 2001; Sheir et al., 2010; Sheir and Handy, 2010).

Table 2: Some examples of metal toxicity in bivalves

Species	Chemicals/ lab or field	Concentration ($\mu g\ l^{-1}$)	Exposure time	Sub lethal responses	References
Mussel, *Mytilus edulis*	Hg (Lab)	20 or 50	11 days	Transient increase in phagocytosis, no effect on NRR or cytotoxicity	Sheir et al. (2010)
Mussel *Mytilus edulis*	Cd (Lab)	20 or 50	11 days	Transient modulated phagocytosis, 3.6 fold time-dependent increase in NRR uptake, no effect on cytotoxicity. Histological alterations in the gills, digestive glands.	Sheir and Handy (2010)
Clam, *Tapes philippinarum*	Cu (Lab)	10, 60, 110	7 days	Decreased phagocytotic activity, increased NRR uptake, reduction in SOD activity	Matozzo et al. (2001)
Clam, *Tapes philippinarum*	Cd (Lab)	150,300, 600	7 days	Increased NRR uptake, no inhibition of phagocytotic activity, no changes in SOD activity	Matozzo et al. (2001)
Mussel, *Mytilus galloprovincialis*	Cd (Lab/ field)	200	7 days in aquaria followed by 28 days detoxification in sea	Induction of MT. Mussel later exposed to 300 $\mu g\ l^{-1}$ Fe caused a Cd- dependent resistance to oxidative stress	Viarengo et al., (1999)
Mussel, *Mytilus edulis*	Cd, (lab)	40 or 400	7 days	Impaired defence capability and increased disease susceptibility	Coles et al. (1995)

1.3.5. Toxicity of Mixtures of Metals

Seldom are organisms exposed to a single contaminant in the aquatic environment, rather organisms are exposed simultaneously to combinations of contaminants which may interact and mutually influence toxicity (Luoma, 1983; Bryan and Langston, 1992; Landis and Yu, 1995; Stewart, 1999). The behaviour of metals in mixtures may not correspond to that predicted from data on the pure metal alone (Landis and Yu, 1995; Altenburger et al., 2003; Norwood et al., 2003; Kamo and Nagai, 2008). The combined effects may be additive (mixtures toxicity is similar to the sum of the toxicities on each metal), synergistic (mixtures of metals showed increased toxicity beyond the simple sum of the toxicities of each metal) or antagonistic (mixtures of metals are less toxic than the sum of the toxicities of each metal) (Landis and Yu, 1995; Altenburger et al., 2003; Kamo and Nagai, 2008). The additive effect could be derived from the concepts of dose (or concentration) addition and independent action. Additive effect could also be calculated by building the sum of the individual metal effect of all the mixture (Kortenkamp and Hass, 2009). Metal mixtures may also influence metal bioavailability and bioaccumulation depending on the metal concentrations, relative binding strengths of the metals for various substrates (partitioning coefficients), and the physiological roles of the metals (Stewart, 1999). The interactions of the components in a mixture can cause complex and substantial changes in the apparent properties of its constituents (Altenburger et al., 2003).

1.4. Choice of Test Chemicals

Two chemicals, mercury and cadmium (salt forms), listed among the priority substances as defined by Water Framework Directive (WFD), Directive 2000/60/EC, were selected for this study. Hg and Cd have been extensively studied and are non-essential, toxic, persistent and accumulating metals (Morel et al., 1998, Rainbow and

Black, 2005). Hg and Cd are transition metals and belong to the group 12 formerly known as group II b (old IUPAC numbering) in the periodic table hence, the similarities in chemical reactivity. In nature, Hg and Cd occur in the earth's crust associated with zinc and copper ores which can be released through natural processes such as weathering of parent rock into the environment creating background levels (Cheng et al., 2014; UNEP, 2013). The most significant sources of the total amount of Hg and Cd entering into environment are from atmospheric emissions from anthropogenic sources e.g., incineration of municipal solid waste (MSW), combustion of coal, and production of non-ferrous metals (Nriagu, 1989; Cheng et al., 2014; UNEP, 2013). The total concentration of metals in water bodies are highly variable, for total Hg concentrations, values ranges from < 5 ng Hg l^{-1} in unpolluted surface water (Cossa and Fileman, 1991) to levels as high as 50 µg Hg l^{-1} in water bodies near industries discharging Hg (Rocha et al., 2013). For total Cd concentration, values ranges from as low as < 5 ng Cd l^{-1} (WHO 1992) to as high as 100 ng Cd l^{-1} near coastal areas (Elinder, 1985), (see Chapters 3 (Cd) and 4 (Hg) for detailed and specific toxicity on mussels).

1.5. Test Organism

1.5.1. The blue mussel

The blue mussels belong to the Mytilus genus (*M. edulis, M. galloprovincilais, M. trossulus*). They are found world-wide and are important in coastal ecology. Mussels are widely used for ecotoxicological studies and have been well established as an indicator of aquatic contamination (Goldberg et al., 1978; Dhawan et al., 2009). The wide distribution, availability, easy accessibility and handling qualities of the mussels have certified them as a model organism in scientific research for exploring metal toxicity (Goldberg et al., 1978; Phillips and Rainbow, 1993).

The mussels are intertidal organisms. By default, mussels have behavioural and physiological adaption to intermittent exposure lifestyle (Helm and Trueman, 1967; Coleman and Trueman, 1971). Mussels occur from the high intertidal to the shallow subtidal attached in clumps by fibrous byssus threads to suitable substrata (Tyler-Walters, 2008). Owing to the tidal habitat, mussel experience at least one pulse (out of seawater) per day. The sedentary, sessile, and filter feeding lifestyle make mussels good detectors of "early warning" of coastal contamination (Dyrynda et al., 1998). The uptake and accumulation of metals by mussels are usually directly proportional to ambient concentrations (Phillips, 1980; Livingstone and Pipe, 1992). Examples of responses to enhanced contamination stress in mussels include immunotoxic, genotoxic, cytotoxic as well as histopathological alterations (Coles et al., 1995; Al-Subiai et al., 2009; Sheir and Handy, 2010; Sheir et al., 2010; Al-Subiai et al., 2011)

1.5.2. Verification of the Mytilus galloprovincialis

The *Mytilus* species used in the present study was verified as *M. galloprovincialis* based on previous reports in the literatures. Firstly, verification was based on species distribution. According to Skibinski et al. (1983) the distribution of the different species of mussels is mosaic with hybridisation occurring between parents at the population boundaries. *M. edulis* is found in the Northern latitudes (e.g., Scotland, Northern/ mid England) while *M. galloprovincialis* found further south (part of Southern England, Atlantic France and the Mediterranean) (Bierne et al., 2003; Hilbish et al., 2002: Skibinski et al., 1983). In the Southwest England, *M. edulis and M. galloprovincialis* has been reported to co-occur (Hilbish et al., 2002). However, pure populations of *M. galloprovincialis* have been reported in North Cornwall, northwest of St. Ives (Hilbish et al., 2002), Port Quin (Gilg and Hilbish, 2003; Craft et al., 2010).

Hines et al (2007) reported no evidence of hybrids between *M. galloprovincialis* and *M. edulis* in Port Quin, North Cornwall.

Secondly, with the use of Me-15 and Me-16 primers, mussels from Port Quin have been established as *M. galloprovincialis* (Hilbish et al., 2002; Craft et al., 2010). Based on these evidences, it could be concluded that the mussels used in this study are likely to be *M. galloprovincialis* (Lamarck).

1.6. Hypotheses

This study was designed to compare the accumulation and sub-lethal physiological effects of two bioaccumulative metals exposed singly or in mixtures to mussel during intermittent and continuous exposures. The main hypothesis is that accumulation and biological responses of *M.galloprovincialis* exposed to Hg and Cd singly or in mixtures during continuous exposure will be different from the intermittent counterparts. The target organs may be the same but the processes of toxicokinetics (absorption, distribution, metabolism and excretion) and toxicodynamics (what the metal does at the site of action and its effect) may be different between the two exposure regimes. Overall, this thesis tested the following hypotheses.

I. Tissue metal concentration and responses during intermittent exposure will be half of those of the continuous counterpart.

II. Metal accumulation during the Hg plus Cd mixture will be additive as metal uptake will be from independent pathways. Responses in the mussel will also be the additive since both metals have similar mode of action

1.7. Aim and Objectives

The overall aim of the experiment was to investigate the bioaccumulation responses of two bioaccumulative metals exposed to *M.galloprovincialis* singly or in mixtures during intermittent exposure compared to the continuous counterpart.

1.7.1. Specific Objectives

I. To compare the bioaccumulation responses in the tissues (adductor muscles, digestive gland, gill, gonad and remaining soft tissues within the shell) of *M. galloprovincialis* during continuous and intermittent exposure of single metal, cadmium or mercury (Chapters 3 and 4).

II. To compare the bioaccumulation responses of metals (Cd and Hg) mixture in the tissues (adductor muscles, digestive gland, gill, gonad and remaining soft tissues within the shell) of *M.galloprovincialis* during continuous and intermittent exposure to metals mixture, Hg plus Cd (Chapters 5 and 6).

Chapter 2 *:*

General Methodology

2.0. Chemicals

All chemicals were obtained from Sigma Aldrich (Poole, UK) and Fisher Scientific Ltd (Loughborough, UK) except otherwise stated.

2.1. Sampling Site of Test Organisms

Port Quin harbour was chosen as the site of blue mussels, *M. galloprovincialis* collection for the experiments, based on previous experiments in our laboratory (Sheir and Handy, 2010; Sheir et al 2010). Port Quin (Grid Reference, SW97150805; latitude 50º 35' 21.67'' N, longitude 04º 52' 1.7''W) is a small cove and hamlet between Port Isaac and Polzeath, a rural area with no industrial input, located on the Atlantic coast in north Cornwall, UK (Figure. 1)

2.2. Collection and Maintenance of Test Organism

Adult mussels, *M. galloprovincialis* selected by valve length (40-60 mm) were collected and immediately transported (approximately 2 h) in a cool box to the Plymouth laboratory. Thereafter, mussels were cleaned of field debris and placed in a plastic trough containing filtered (0.22 µm mesh size) natural seawater (collected from Mount Batten breakwater in Plymouth Sound at high tide around a period of low rainfall and stored in tanks under the ground at ambient temperature at the Plymouth laboratory) with constant aeration. Stock mussels were kept in a controlled temperature (15 ± 1 ºC) room under a photoperiod of 12 h light: 12 h dark. Stock mussels were initially allowed three days with no food but daily water change to depurate food and particulate matter from the field. Thereafter, mussels were fed twice weekly on

commercial instant algae (Reed Mariculture Inc, USA) and allowed to acclimatize for at least two weeks. Stock water was renewed twice a week.

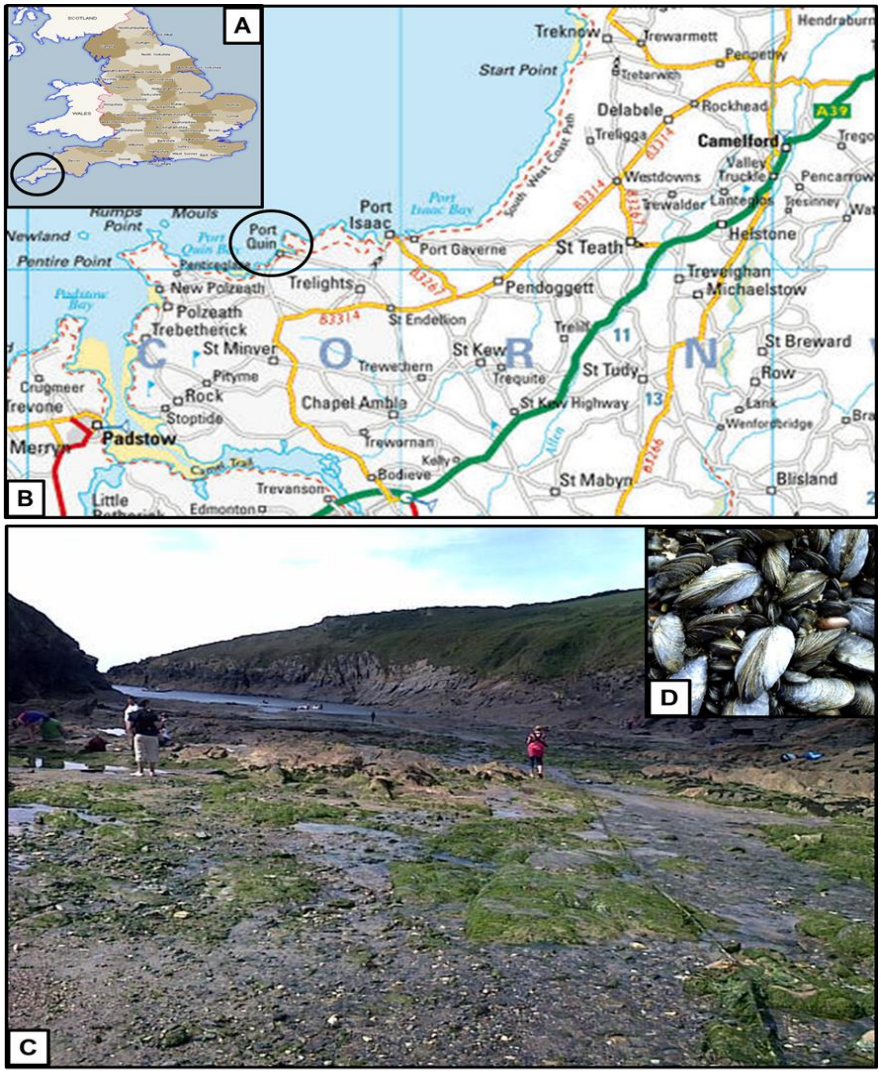


Figure 1: Port Quin (mussels sampling site)

(A) Map of United Kingdom showing Cornwall (black circle).
(B) Map showing Port Quin (black circle).
(C) Port Quin sampling site.
(D) Aggregate of *M.galloprovincialis* attached to substrate.

2. 3. Experimental Design

The experimental design for mussels exposed to metals (Cd and/or Hg) involved triplicate glass tanks for each treatment, with appropriate clean filtered seawater water controls. All glass tanks were acid soaked for three days with 2% nitric acid double rinsed with deionised water and dried prior to use. For the Cd alone (Chapter 3) and Hg alone (Chapter 4) experiments, mussels were exposed in a triplicated design (3 tanks/treatment) for 14 days using a semi-static exposure regime to control (filtered seawater only), or 50 µg l^{-1} of either Cd as $CdCl_2$ or Hg as $HgCl_2$ for both continuous and intermittent exposures (Figure. 2). In the mixture (Cd and Hg combined) experiments, mussels were exposed in triplicate design for 14 days using a semi-static exposure regime to four treatments (i) filtered seawater only control; (ii) 50 µg l^{-1} Cd alone control; (iii) 50 µg l^{-1} Hg alone control; equal concentrations of 50 µg l^{-1} Cd and Hg for both continuous (Chapter 5) and intermittent (Chapter 6) exposures. The exposure concentration (50 µg l^{-1}) of Hg or Cd was selected based on previous experiments in our laboratory (Sheir and Handy, 2010; Sheir et al., 2010). Sheir and Handy (2010) and Sheir et al. (2010) performed a range finding test on *M. edulis* using different concentration of Cd (0-50 µg l^{-1}) and Hg (0-500 µg l^{-1}). The aim was to identify the concentration where mussels will accumulate metal without any mortality or closure of the valve. The concentration (50 µg l^{-1}) was chosen as the ideal concentration for sub-lethal bioaccumulation responses for both Cd and Hg. Dosing was achieved by adding 1 ml of a 1 g l^{-1} Cd or Hg stock solution to tanks containing 20 l of filtered seawater for the individual metals experiment (Chapters 3 and 4), or 0.5 ml of 1 g l^{-1} Cd and/or Hg stock solution to tanks containing 10 l filtered seawater for the combined experiments (Chapters 5 and 6) to give a nominal concentration of 50 µg l^{-1} for Cd and Hg each. The continuous exposure tanks were dosed daily while the

intermittent exposure tanks were dosed and filled with clean filtered seawater alternately every 2 days.

To maintain more than 80 % of the starting concentration, tanks were covered throughout the exposure period (Figure 2). Complete water changes were also done daily; tanks were double rinsed with clean filtered seawater and re-dosed after each water change. Mussels were not fed 24 h prior to, or during the experiments, in order to minimise the risk of the metal adsorption to food or faecal material and to maintain water quality throughout the exposure period. Water samples were collected daily during both the acclimation and exposure periods for water quality parameters including; pH (pH 301, Hanna Instrument, pH/ ion meter, Leighton Buzzard, UK), salinity (YSI 63, refractometer, Fleet, UK), dissolved oxygen (Hach Lange LDO-HQ 10 DO meter, Salford, UK) and, total ammonia (HI 95715, Hanna Instruments). For trace metals concentrations, water samples were collected daily immediately before and after renewal of the test medium from individual tanks.

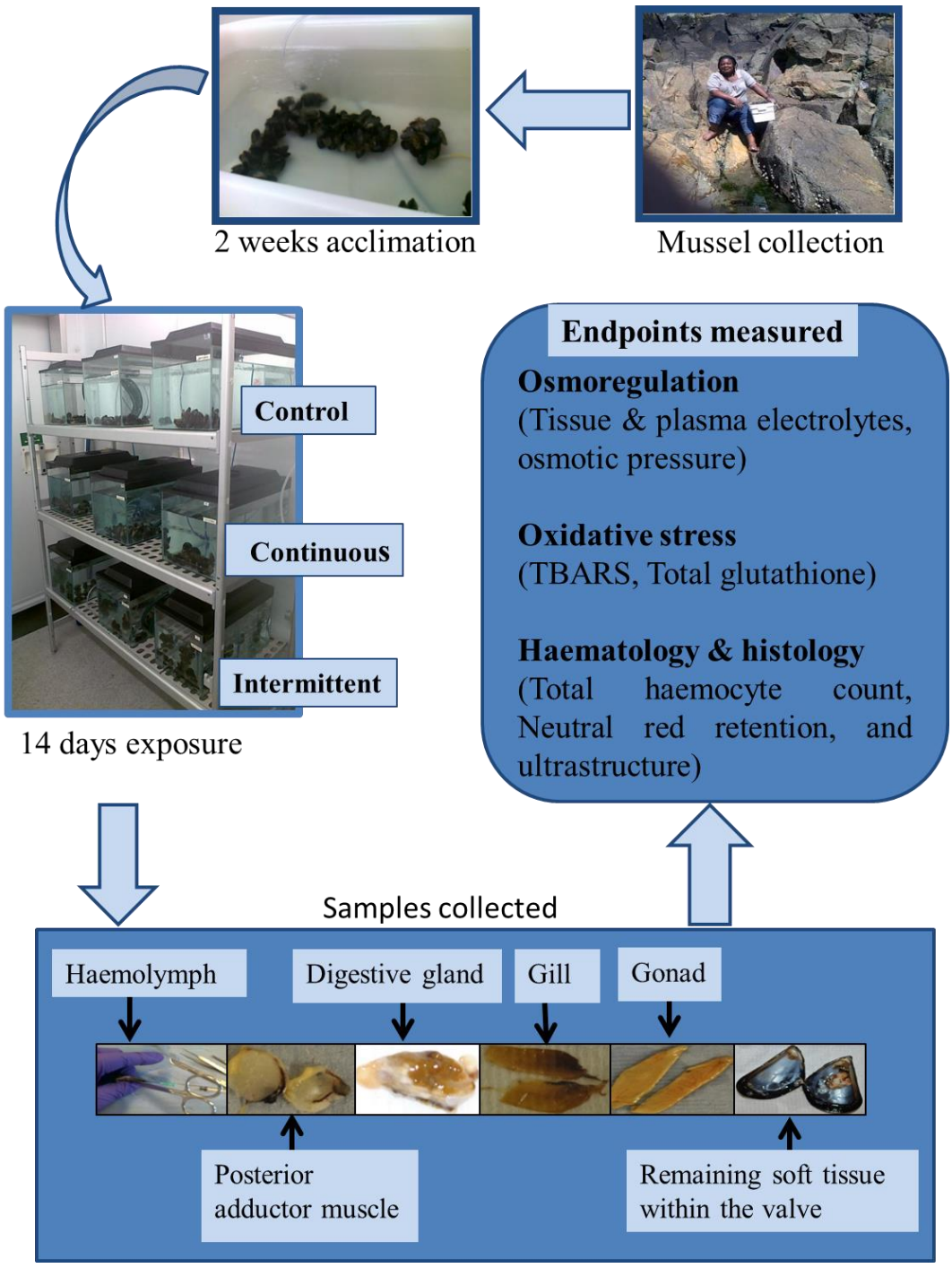


Figure 2: An overview of the experimental design for Cd alone (Chapter 3) and Hg alone (Chapter 4) during continuous and intermittent exposures. *M. galloprovincialis* were collected from the field, thereafter acclimated for 2 weeks in the laboratory. Mussels were exposed for 14 days in a triplicated design to control (no added Hg or Cd), or to continuous and intermittent exposure (50 µg l^{-1} of either Cd or Hg). Tissue and haemolymph samples were collected and endpoints were measured.

2. 4. Haemolymph Extraction and Tissues Collection

On sampling days, mussels (2/tank or 6/treatment) collected from the experimental tanks were first rinsed in clean filtered seawater to remove excess metal on the valves. The valves were then slightly opened to allow, firstly, for complete draining of seawater, and secondly, for easy access to the posterior adductor muscle for the extraction of haemolymph. Haemolymph (0.4-1 ml) was then withdrawn from the posterior adductor muscle with a clinical syringe (1 ml) attached to a hypodermic needle (21G) and transferred to Eppendorf tubes placed on ice. Haemolymph required for the measurement of osmotic pressure, electrolytes and glucose concentration were allowed to settle gravitationally (at least 2 hours) and the cell-free haemolymph (plasma) stored at -80 °C for later analysis (Sections 2.11 and 2.12). Whole haemolymph was used for the neutral red retention assay (only Chapters 3 and 4), haemolymph protein (Chapters 5 and 6) and total haemocyte counts. Mussels were thereafter dissected to collect tissues (posterior adductor muscle, digestive gland, gill, gonad, and the remaining soft tissues within the valves, except for the byssus threads). Byssus threads were not collected due to the size differences between individual mussels. Clean, acid-washed instruments were used to avoid cross-contamination between treatments and tissues. Wet tissues were rinsed with ultrapure water (Milli-Q), and blotted with a tissue to remove excess water. Tissues were thereafter placed in pre-weighed scintillation vials (20 ml) and oven-dried to a constant weight at 60 °C. Additional mussels (2/ tank or 6/ treatment) were dissected for biochemistry and histology. Half of the tissues (posterior adductor muscle, digestive glands, gills and gonads) excised from the additional mussels were immediately snapped frozen in liquid nitrogen and stored in -80 ˚C until required for biochemistry (Section 2.7). The other half of the tissues (gill and digestive gland) were placed in individual vials containing formal saline (9 g NaCl, 100 ml of 40%

formaldehyde, pH 7.4, made up to 1 l with Milli-Q water) for histological examination (Section 2.6).

2. 5. Trace Metal Analysis

Tissues, haemolymph and water were analysed for trace elements concentrations as described in Sheir and Handy (2010). Briefly, digested tissues (posterior adductor muscle, digestive gland, gill, gonad, and the remaining soft tissues within the valves, except for the byssus threads) were oven dried to a constant weight and digested in concentrated nitric acid for 2 hours at 70 °C. After this they were, allowed to cool, and thereafter diluted with ultrapure (Milli-Q) water to a final volume of 5 or 20 ml depending on the dried tissue weight; ≤ 0.1 g or > 0.1 g, respectively. Tissue metal (Cd or Hg) and electrolyte (Na^+, K^+, Ca^{2+} and Mg^{2+}) concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725-ES, Melbourne, Australia), while the water and haemolymph samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific XSeries 2, Hemel Hempstead, UK). Calibrations for ICP-OES and ICP-MS were performed with matrix-matched analytical grade standards containing internal reference materials indium and iridium; for tissue (0.5%, ICP-OES), water (0.5%, ICP-MS) and haemolymph (1% ICP-MS).

2. 6. Histological Examinations

To determine contaminant-specific alterations in the tissues, histological examinations were also conducted at the end of the experiment. After fixing in formal saline (9 g NaCl, 100 ml of 40% formaldehyde, pH 7.4, made up to 1 l with Milli-Q

water), tissues from all treatments (control(s), continuous and intermittent exposures) were processed together using an automated processor (Leica TP1020 tissue processor, nussloch, Germany) using an established dehydration and paraffin infiltration protocol for small invertebrate tissue (Table 3). After processing, tissues were transferred to an embedding machine (Leica EG1150H, Germany) and paraffin wax blocks prepared manually and left to harden on the cold plate of the embedding machine.

Table 3: Protocol for dehydration and paraffin infiltration of small invertebrate tissue

Step	Reagent	Duration (h: min)
1	70% ethanol	Overnight
2	90% ethanol	2
3	100% Industrial methylated spirit (IMS)	2
4	Absolute ethanol	2
5	Absolute ethanol	Overnight
6	[1]Histoluene	1
7	Histoluene	:30
8	Histoluene	:30
9	[2]Wax (65 ºC)	1
10	Wax	:30

[3]Histology guide by Hockings, M., Bloomfield, S.M and Russel, P

[1] Histoluene (CellPath Ltd, UK).
[2] Wax , Cellplus polymer added (CellPath Ltd, UK)
[3] Plymouth University Histology guide (unpublished material)

The blocks were kept until required for sectioning. Transverse sections (5-8 µm thickness) were cut with a microtome (Leica RM2235, Germany) and placed first in 30% ethanol, then transferred to the surface of water maintained at 50 °C in the paraffin section mounting water bath (Electrothermal MH8514, England). The ribbons were allowed to expand to full size. Sections were picked out of the water with a microscope slide and allowed to dry. After drying, slides were stained manually with haematoxylin and eosin (Mayer's H and E) using an established staining protocol (Table 4). Staining was done in batches with slides from all treatments to eliminate artefacts between treatments. After staining, a few drops of DePeX mounting solution were placed on the slides to enhance adherence of the coverslip. Histological examination was performed using Olympus light microscopy (Vanox-T, AH-2, Japan) and photographs were taken (Olympus digital camera, C-2020 Z). Identification of histological changes on the slides was based on previous work in this laboratory (Sheir and Handy, 2010). For the digestive gland, the proportion of the tubule area, connective tissue as well as the fractional area of the connective tissue infiltrated by haemocytes were counted with the differential point counting method and calculated with the formula: $Vi = Pi/P_T$, where *Vi* is the volume fraction, *Pi* is the number of points counted and P_T is the total number of points on the counting lattice (Weibel et al., 1966). For epithelium cell height, 10 tubules were randomly selected from each section and manually measured. The number of digestive tubules with injury was also counted in each section. In the case of gill injury, a range of 50-80 gill filaments was counted and filaments with injury identified and counted.

Table 4: Protocol for Haematoxylin and Eosin staining.

Station	Reagent	Time (min:s)
1	Histoluene	02:00
	Histoluene	02:00
3	Absolute ethanol	02:00
4	Absolute ethanol	02:00
5	90% ethanol	02:00
6	70% ethanol	02:00
7	50% ethanol	02:00
1st wash	Tap Water	02:00
6	Mayer's Haematoxylin (see below)	25:00
2nd wash	Tap Water	05:00
7	Lithium Carbonate ($LiCO_3$)	00:01
3rd wash	Tap Water	02:00
8	Acid ethanol (70% ethanol plus 1% concentrated Hydrochloric Acid)	00.02
4th wash	Tap Water	02:00
14	Eosin (1% in 1% calcium nitrate)	00:30
5 wash	Tap Water	05:00
16	90% ethanol	00:30
17	Absolute ethanol	02:00
18	Absolute ethanol	02:00
13	Histoluene	02:00
9	Histoluene	02:00
Exit	Histoluene	

[4]Histology guide by Hockings, M., Bloomfield, S.M and Russel, P

[4] Plymouth University Histology guide (unpublished material)

2.7. Oxidative Stress

Oxidative stress occurs when the rate of reactive oxygen species (ROS) production overwhelms the endogenous protection afforded by specific degradative enzymes, antioxidant vitamins, and other radical scavengers in the organism. Contaminant-induced ROS with subsequent oxidative damage has been proposed as a mechanism of toxicity in aquatic organisms (Livingstone, 2001). In the present study, two standard assays used in determining oxidative damage were employed: thiobarbituric acid reactive substances, TBARS (marker for the available thiols in a tissue homogenate) and total glutathione, reduced (GSH) and oxidised (GSSG) (the non-enzymatic antioxidant scavenger).

2.7.1. Thiobarbituric Acid Reactive substances, TBARS

Half of the tissues (posterior adductor muscle, digestive gland, gills and gonads) excised from each mussel (see Section 2.4) were immediately snapped frozen in liquid nitrogen and stored in -80 °C until required. Tissues (about 0.1 g) were homogenised (Cat X520D homogeniser with a T6 shaft, medium speed, Bennett and Company, Weston-super-Mare) at full speed (18,000 *RPM*) in 5 volumes of ice-cold buffer (20 mmol l^{-1} 4-(2-hydroxylmethy) piperazine-1-ethane sulfonic acid (HEPES), pH 7.8 (with Tris, 2-amino2-hydroxymethyl-1, 3-propanediol) containing 300 mmol l^{-1} sucrose and, 0.1 mmol l^{-1} ethylenediamine tetra acetic acid (EDTA). Tissues were then centrifuged at 13000 *RPM* (12,303 $_{x}g$) for 2 min and the supernatants were stored in aliquots at -80°C until required for analysis.

Thiobarbituric acid reactive substances such as the lipid hydroperoxides and aldehydes are naturally present in biological samples (Botsoglou et al., 1994). Lipid peroxidation (oxidation of lipids) is a well-defined mechanism of cellular damage and is

used as an indicator of oxidative stress (Botsoglou et al., 1994). The use of the TBARS assay to measure the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage (Camejo et al., 1998; Yagi, 1998).

In the present study, the TBARS assay was used to determine the total lipid peroxidation in tissue samples. The TBARS assay is a colorimetric assay based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, TBA with aldehyde to yield a chromophore (1 aldehyde: 2 TBA adduct) under high temperature and acidic conditions with an absorbance maximum at 530 nm. The assay was performed as described in Federici et al. (2007) with slight modifications. The assay was first performed in Eppendorf tubes (for easy centrifugation) and then transferred to 96-well microplates for reading. Forty microliters of tissue (adductor muscle, digestive glands, gill and gonads) supernatants, standard or blank (triplicate) were placed in Eppendorf tubes (in triplicate) already containing 10 µl of 1 mmol l^{-1} 2, 6 -Di- 0- tert-butyl-4-methylphenol dissolved in ethanol to stop further oxidation of the samples. Then, 140 µl of 0.1 M sodium phosphate buffer (pH 7.5) was added to each tube, followed by 50 µl of 50% (w/v) trichloroacetic acid (TCA) and 75 µl of 1.3% (w/v) thiobarbituric acid (TBA, Sigma, T5500) dissolved in 0.3% (w/v) sodium hydroxide solution (NaOH). The Eppendorf tubes were then incubated at 60 ºC for 1 h, allowed to cool and centrifuged at 13,000 *RPM* (12, 303 $_{x}g$) for 2 min. Thereafter, 200 µl of the supernatant from each sample, standard and blank were transferred into the 96-well plate for spectrophotometry. Absorbance was read in a plate reader (VERSA max tunable micro plate reader, Molecular Devices, USA) using the software Softmax® Pro 5, first at 530 nm, and then at 630 nm to correct for turbidity. The concentration of TBARS was determined from a standard curve (0-150 µmol l^{-1}, 1,1,3,3-tetra ethoxy propane, Figure 3), and data expressed as TBARS nmol mg^{-1} protein.

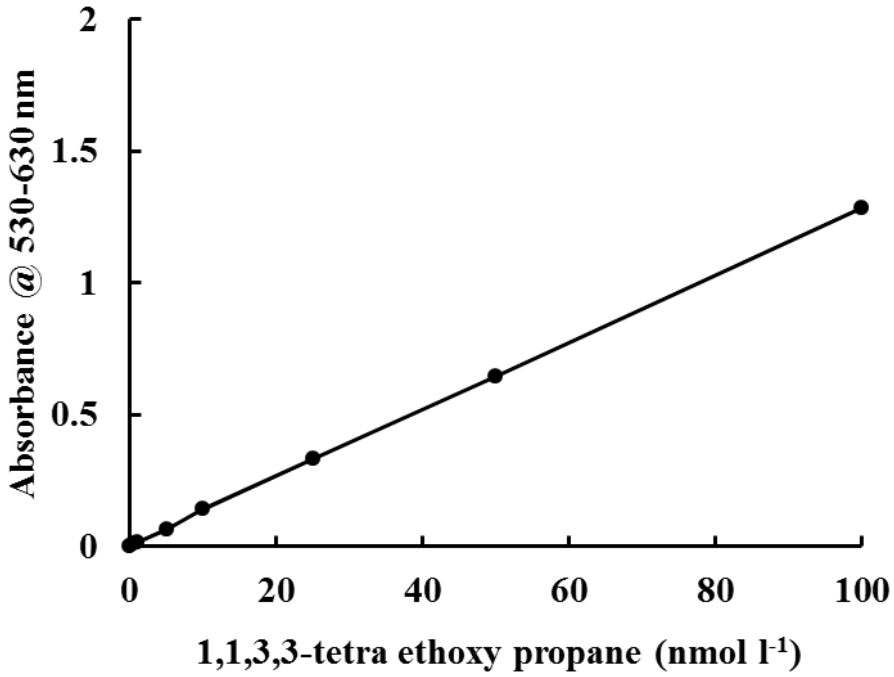


Figure 3: Example of the 1,1,3,3-tetra ethoxy propane standard curve. The absorbance was measured at 530 nm and corrected for turbidity at 630 nm. The corrected absorbance (530-630 nm) was described by a linear regression ($y = 0.0128x - 0.0063$; $r^2 = 0.9999$). Each point represents the mean ± SEM, $n = 3$.

2.7.2. Total Glutathione

Glutathione (GSH) is the most abundant cellular thiol and is involved in metabolic and transport processes as well as in the protection of cells against the toxic effects of compounds such as ROS and heavy metals by acting as an antioxidant scavenger (Canesi et al., 1999, Livingstone, 2001). The measurement of the GSH and its disulfide form, glutathione disulfide (GSSG), provides information about cellular defence and cellular response to stress. Alterations in glutathione concentrations have been used as a marker for oxidative stress in biological samples (Baker et al., 1990).

In the present study, total glutathione (i.e., reduced, GSH and oxidised, GSSG) of the tissue supernatants was determined using the method based on the Owens and Belcher (1965) cyclo-reduction method (Figure 4). The method uses a kinetic reaction

in which oxidised glutathione (GSSG) is reduced enzymically by glutathione reductase (GR) in the presence of NADPH to reduced glutathione (GSH). The GSH then reacts with 5,-5'-dithiobis-(2-nitrobenzoic acid), DTNB (Ellman's reagent) to form 5-thionitrobenzoic acid (TNB, an intense yellow colour product that can be quantified spectrophotometrically at the 412 nm) and a mixed disulphide (GSSTNB). The GSSTNB reacts with further quantities of GSH to liberate another TNB and GSSH, which is then recycled (Figure 4). The assay uses a single standard (20 µmol l^{-1}) of reduced glutathione (GSH) and a blank (Mili-Q) in triplicate design per run to calibrate the results. Briefly, tissue (posterior adductor muscle, digestive gland, gill and gonad) supernatants, standard (20 µmol l^{-1} reduced glutathione solution) or blank (only Milli-Q water) were mixed in a 1:1 ratio with 10 mmol l^{-1} 5,5'-dithiobis-(2-nitrobenzoic acid, DNTB (Sigma, D8130) freshly prepared in assay buffer (100 mmol l^{-1} potassium phosphate, pH 7.5, containing 5 $mmol^{-1}$ EDTA). Forty microlitres of the DTNB-supernatant mixture, GSH standard or blanks were placed in a 96-well plate. Two hundred and ten µl of assay buffer containing 0.6 U ml^{-1} glutathione reductase (from baker's yeast, Sigma G-3664) were then added to each well, mixed and equilibrated for 1 minute. The reaction was started by the addition of 60 µl of 1 mmol l^{-1} NADPH (Melford Laboratories Ltd, Ipswich, UK), and rate of change in absorbance at 412 nm recorded over periods at least 5 min in a plate reader (VERSA max tunable micro plate reader, USA) using the software Softmax® Pro 5. Total glutathione concentrations in the tissue supernatants were calculated with the following equation 1 and expressed as nmol g^{-1} wet weight:

Equation for total glutathione calculation: Equation 1

$$\text{Total glutathione (nmol g}^{-1}\text{ wwt)} = \left[\frac{\Delta 412\text{ (sample)} - \Delta 412\text{ (blank)}}{\Delta 412\text{ (standard)}}\right] \times 20\ \mu\text{mol l}^{-1} \times \text{tissue wt (g)} \times \text{DF}$$

Where, DF = dilution factor; Δ = rate of change of absorbance (nm); wwt = wet weight.

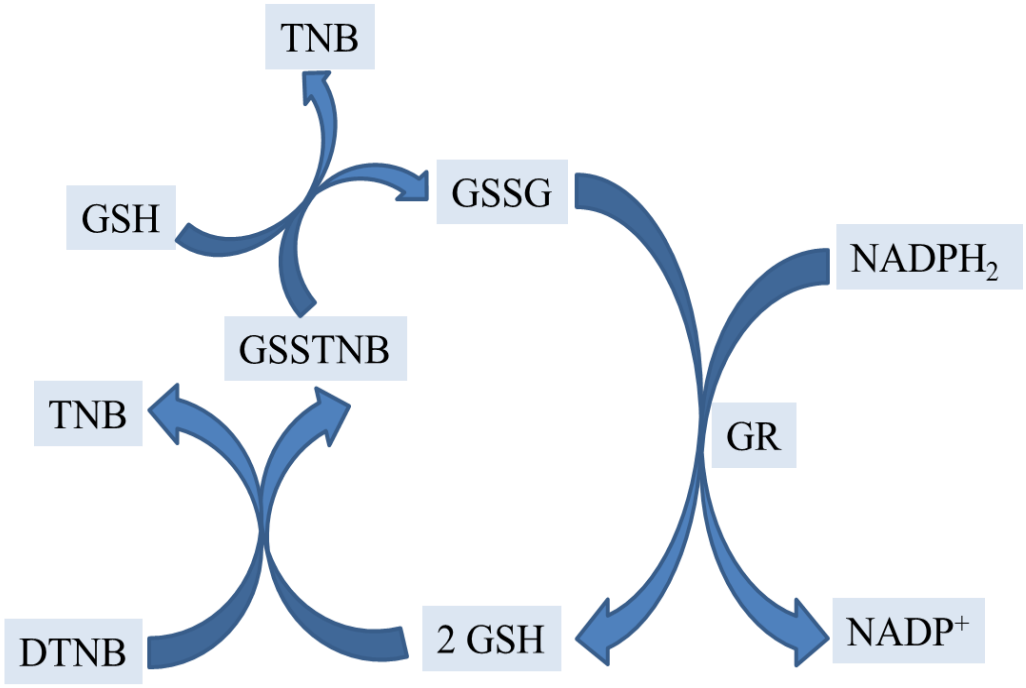


Figure 4: Owens and Belcher cyclo-reduction method. GSSH is reduced enzymically by glutathione reductase (GR) in the presence of NADPH to GSH, which then reacts with DTNB to form TNB an intense yellow colour product and a mixed disulphide (GSSTNB). The GSSTNB reacts with further quantities of GSH to liberate another TNB and GSSH, which is then recycled.

2.8. Protein Analysis

Protein concentrations were determined by the Bradford method using a commercial kit (Sigma reagent: Bradford, B6916). The Bradford assay is a colorimetric protein assay based on the interaction between the proteins in the sample and the Coomassie Brilliant Blue G-250 dye. The protein-dye complex formed causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present in the samples. Briefly, 5 µl of samples (tissue or haemolymph) standards, or blank, or samples were plated in triplicate. Two hundred and fifty microlitres of the Bradford reagent (not diluted) was added and the colour allowed developing for 30 min in the dark (foil) at room temperature. Absorbance was read at 595 nm in the plate reader (VERSA max tunable micro plate reader, above) against 0-1 mg ml^{-1} bovine serum albumin standards (Figure 5).

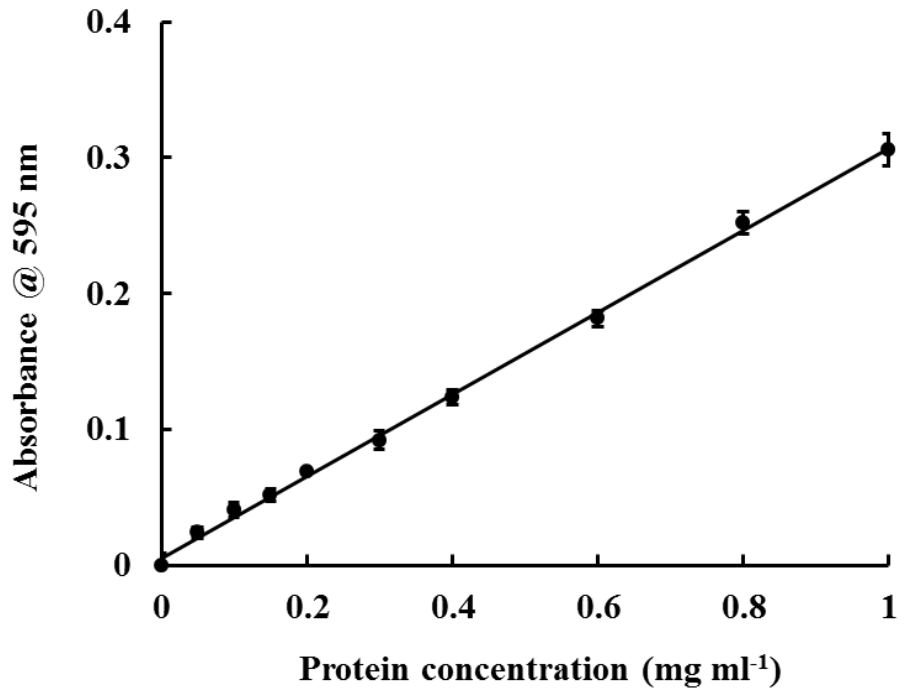


Figure 5: Example of the bovine serum albumin (protein) standard curve. The absorbance was measured at 595 nm and was described by a linear regression (y = 0.3013x + 0.0054; r^2 = 0.9984). Each point represents the mean ± SEM, n = 3.

2. 9. Neutral Red Retention (NRR)

The neutral red retention assay has been widely used as a good indicator of cell health. Neutral red retention is based on the ability of viable cells to incorporate and bind the neutral red dye in the lysosomes (Lowe et al., 1995). NRR ability of the haemocytes from *M. galloprovincialis* was measured according to Sheir and Handy (2010). Briefly, 50 µl of whole haemolymph was collected from the ice-cold haemolymph described above (Section 2.4) and placed in a 96-well flat bottom microplate. The plate was already pre-coated with 10% (v/v) poly-L-lysine to enhance haemocyte adhesion. The plate was covered and agitated at 400 *RPM* for 1 min, then incubated at 15 °C for 50 min to form a haemocyte monolayer. After the incubation period, the excess haemolymph was carefully removed, then 200 µl of 0.004% (0.004 g of neutral red dye totally dissolved in 100 ml of physiological saline, pH 7.36) neutral red solution was added to each well and the cells incubated in the dark for 3h at 15˚C. After incubation, the supernatant was removed and the cells washed once with 200 µl physiological saline. Two hundred microlitres of acidified ethanol was added to each well to solubilise the neutral red dye. The plate was thereafter incubated in the dark (foil) at room temperature for 10 min. The plate was agitated for 30 s and absorbance read at 550 nm in a plate reader (VERSA max tunable micro plate reader, USA) using the software Softmax® Pro 5. The neutral red retention ability of the haemocytes was expressed as absorbance 10^6 $cells^{-1}$.

2.10. Total Haemocyte Counts (THC)

Haemocyte counts were performed according to Coles et al. (1995). Briefly, whole haemolymph was immediately fixed in an equal volume of Bakers formal calcium (BFC; 1% calcium acetate, 2% sodium chloride and 4% formaldehyde made up

to 100 ml in Milli-Q water) and counted on a Neubauer Improved haemocytometer afterwards under an Olympus microscope (CK30-F200, Japan).

2.11. Determination of Plasma Ions and Osmometry

Na^+, K^+ and osmotic pressure were measured to investigate the effect of metal (s) on the osmotic and ionic regulations in the cell-free haemolymph of mussel. Plasma electrolytes (Na^+ and K^+) were analysed by flame photometry (Sherwood 420 flame photometry, UK). Briefly standards (100 $mmol^{-1}$, NaCl and KCl) and samples (20 µl) were prepared in 100 mg l^{-1} lithium chloride and results expressed in mmol l^{-1}. The osmotic pressure measured as osmolality was determined in 50 µl of plasma with the freezing-point depression osmometer (Gonotec Osmomat 030, Cryoscopic osmometer, Germany). Calibrations were made with a 1000 mosmol Kg^{-1} standard from the same manufacturers (Gonotec Osmomat 030).

2.12. Determination of Plasma Glucose

Alterations in plasma glucose levels have been used as an indicator of starvation induced stress in *M.edulis* (Sheir and Handy, 2010; Sheir et al., 2010). In our study, plasma glucose was measured based on the Braham and Trinder (1972) glucose oxidase method with slight modifications for microplate. The method uses an enzymatic reaction where glucose oxidase first catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2). The H_2O_2 formed then reacts with p-hydroxybenze sulfonate and 4-aminoantipyrine in the presence of horseradish peroxidase to form a quinoneimine dye (Equation 2) that can be quantified spectrophotometrically at 505 nm. The intensity of the colour produced is directly

proportional to the glucose in the sample. Briefly, tissue supernatants were diluted (1:5) with physiological saline. Five microlitres of each diluted supernatant were plated in triplicate wells of 96 well plates. Afterwards, 200 µl of 0.1 M potassium phosphate, pH 7.0 containing 0.5 mmol l^{-1} 4-aminoantipyrine, 20 mmol l^{-1} *p*-hydroxybenze sulfonate, 15, 000 U l^{-1} glucose oxidase (from *Asperigillus niger*) and 10, 000 U l^{-1} of peroxidase (from horseradish) were added and incubated at 22 ºC for 18 min. Plates were shaken for 15 s and the absorbance read at 505 nm (VERSA max tunable microplate reader, USA) against standards (0-2 mmol l^{-1} D-glucose, Figure 6).

The glucose assay principle is as follows: Equation 2

$$\beta\text{ - D - glucose} + O_2 + H_2O_2 \xrightarrow{\textit{Glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{p-hydroxybenzene sulfonate} + \text{4-aminoantipyrine} \xrightarrow{\textit{Horseradish peroxidase}} \text{quinoneimine dye} + H_2O$$

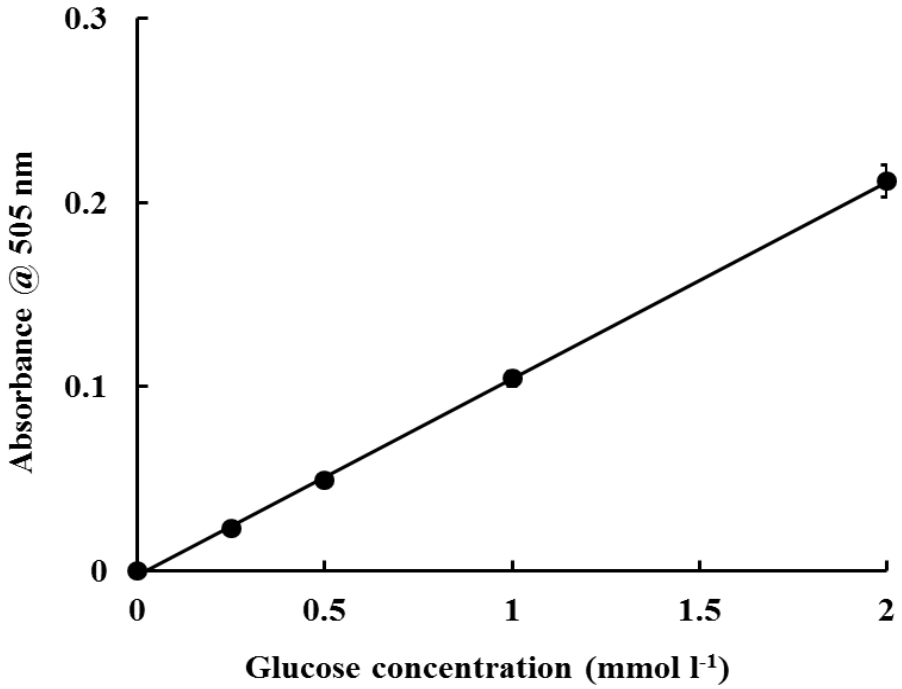


Figure 6: Example of the D-glucose standard curve. The absorbance measured was described by a linear regression ($y = 0.1067x - 0.0024$; $r^2 = 0.9996$). Each point represents the mean ± SEM, $n = 3$.

2.13. Statistical Analysis of Data

Statistical analysis was carried out on all data using Statgraphics Centurion for Windows. No tank effects were observed within treatments so data were pooled by treatment and presented as mean ± standard error of the mean (SEM), for $n = 6$ mussels/ treatment unless otherwise stated. After descriptive statistics on raw and untransformed data, Bartlett's test of homogeneity of variances was performed to verify conformity with the analytical assumptions (equal variance among groups). Where the Bartlett test indicated non-conformity, data were transformed ($\log_{10}$) and the test was performed again. If after transformation, conformity to assumptions of variance homogeneity was met, analysis of variance (ANOVA) was used for the analysis. Data were analysed for treatment and/or time effects by one-way or two-way ANOVA (Table 5). The two-way ANOVA (2-way ANOVA) was used to analyse treatment, time, and treatment × time interactions (Table 6). Where the test showed statistically significant treatment or time effect ($p < 0.05$), the one-way ANOVA was used to test for treatment effects within a time or time effect within a treatment. The Fisher's least square difference test, LSD post hoc test was used at 95% confidence limit to provide specific information on which means are significantly different from each other.

However, if the transformation was not effective (conformity not met), the non-parametric Kruskal-Wallis test was used and significant differences located by the lack of overlap of the notches about the median on the notched Box-and-Whisker plot. Pearson linear correlation coefficient (r) and/or the coefficient of determination (r^2) was used to measure the relationship between the accumulation and responses where appropriate. The detection limit for the metal (s) was derived from three times the standard deviation of the procedural blank.

Table 5: An example of the arrangement of data for statistical analysis

Hg concentration in the gill (ug g^{-1})	Treatments	Time	No of mussels
0.761763775	1	2	1
0.734759433	1	2	2
0.868939123	1	2	3
0.757231623	1	2	4
0.630263316	1	2	5
0.632609117	1	2	6
2.423002298	3	2	1
2.627238078	3	2	2
2.51975129	3	2	3
2.53466641	3	2	4
2.472999553	3	2	5
2.575253987	3	2	6
2.459369034	5	2	1
2.121762217	5	2	2
2.407536909	5	2	3
2.443222635	5	2	4
2.053167297	5	2	5
2.359963085	5	2	6

Treatments 1, 3 and 5 represent control, continuous and intermittent exposure respectively. Time (2) represents the day 2 of exposure to Hg.

Table 6: An example of the 2-way ANOVA for Hg concentration - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Treatments	111.419	2	55.7096	2477.02	0.0000
B: Time	6.95719	6	1.15953	51.56	0.0000
INTERACTIONS					
AB	1.12762	12	0.0939687	4.18	0.0000
RESIDUAL	2.36152	105	0.0224906		
TOTAL (CORRECTED)	121.866	125			

Hg concentration is the dependent variable, the treatments and times are the factors. All F-ratios are based on the residual mean square error.

Chapter 3 ***:***

Comparison of Continuous and Intermittent Exposure to Cadmium in the Blue Mussel, Mytilus galloprovincialis: Accumulation and Sub-lethal Physiological Effects

Abstract

In nature, aquatic contamination is discontinuous with concentrations of chemicals changing over time to give a dynamic rather than a steady-state exposure. Little is known about the bioaccumulation responses of shellfish to metals during intermittent compared to the continuous exposure events. This study aimed to compare Cd accumulation and Cd-dependent physiological responses of mussels, *M. galloprovincialis* during continuous and intermittent exposure. Tissues and haemolymph were collected from *M. gallovincialis* exposed for 14 days to either control (no added Cd, only seawater), or 50 µg l^{-1} Cd as $CdCl_2$ in continuous or intermittent profile (2 day exposure, 2 days in clean seawater alternately), and sub-lethal responses were examined using a suite of assays including total glutathione, TBARS, neutral red retention, total haemocyte counts, osmotic pressure, plasma Na^+ and K^+, plasma glucose and histopathology. Tissue Cd concentration was significantly increased with Cd exposure to either continuous or intermittent mode compared to the control (ANOVA, $p < 0.05$). A time-dependent pattern of accumulation was seen in the continuous exposure group for most tissues, while the intermittent exposure showed step-wise changes in the haemolymph and gonad. Tissue Cd concentration in the continuous exposure was significantly increased ($\geq$ 2 fold) for most tissues compared to the intermittent exposure. No clear differences were seen between the continuous and intermittent exposure for most end points measured apart from a 2 fold significant increase in haemocyte infiltration in the digestive gland of the continuous exposure compared to the intermittent exposure. Overall, the data showed that the accumulation of Cd was higher in continuous exposure; but with less exposure, the intermittent profile produces similar toxicity to the continuous event for most end points in *M.galloprovincialis.*

3.0. Introduction

Intermittent exposures to environmental contaminants are considered to be more environmentally realistic, as organisms are unlikely to be exposed continuously to contaminants in natural ecosystems (see reviews: McCahon and Pascoe, 1990; Handy, 1994). The accumulation and ecotoxicity of metals to aquatic invertebrates has traditionally been assessed using continuous exposure data (Rainbow and White, 1989). For pesticides at least, it has been suggested that water quality criteria for intermittent exposure might be estimated from the No Observable Adverse Effect Concentrations (NOECs) derived from continuous exposure data, with the assumption that response of organisms in intermittent events is equal to those in continuous exposure at an equivalent dose (Boxall et al., 2002). However, there are concerns that toxicity thresholds and NOECs derived from continuous exposure data may not readily apply to intermittent contamination events (Handy, 1994; Ashauer et al., 2007a).

Studies on intermittent exposure to metals are relatively scarce and mainly focus on tissue metal accumulation, with limited or no information on sub-lethal effects or physiological responses. These studies on intermittent exposure include experiments on fishes (Cu, Seim et al., 1984; Handy, 1992; Diamond et al., 2006; Cd, Pascoe and Shazili, 1986; Handy, 1992; Al, Siddens et al., 1986, Zn, Brown et al., 1969; Diamond et al., 2006) or invertebrates (Cd, Coleman, 1980; Zn, Shuhaimi-Othman and Pascoe, 2007) (Tables 1). Fewer studies exploring sub-lethal responses to intermittent exposure have focused on other chemicals. For example, reproductive effects during nitrite (Alonso and Camargo, 2009) or pesticide exposures (Cold and Forbes 2004) and growth rates during pesticide exposures (Jarvinen et al., 1988); changes in osmoregulation during Cu exposures (Davenport 1977). Overall, the responses of the organisms in the above studies were either less or more in the intermittent exposure compared to the continuous counterparts. In addition, there remains (since Handy, 1994) no consensus

view on the relationship between tissue accumulation and toxicity during intermittent exposure.

Cadmium has been extensively studied and is well known for its bioaccumulation potential in aquatic organisms (Rainbow and Black, 2005). Cadmium occurs in the earth's crust and is commonly associated with zinc, lead and copper ores (OECD, 1995). Cadmium is released into the environment through natural and anthropogenic sources. Weathering is the most significant natural source of Cd release. Anthropogenic sources of Cd include smelter emission, sewage sludge, fertilizers, smelting and refining of zinc and lead ores, combustion of coal and oil. Cd occurs naturally in soil and rocks at a concentration of less than 1 μg g^{-1}. In water bodies, the average cadmium concentrations have been reported to be as low as < 5 ng l^{-1} in unpolluted areas (IPCS, 1992) to as high as 110 ng l^{-1} in coastal areas (Elinder, 1985). Both the natural and the anthropogenic sources of cadmium are likely to be released intermittently into the environment. When released into the environment, Cd can be taken up by aquatic organisms.

Most of the works on the sub-lethal effects of Cd in *Mytilus* have used continuous profiles (Redpath and Davenport, 1988; Gardner, 1993; Sheir and Handy, 2010). Very little is known about sub-lethal effects of Cd during intermittent exposure in shellfish and such information have not been documented for *M. galloprovincialis*. The aims of the present study were, firstly, to compare the accumulation of Cd in *M. galloprovincialis* during intermittent and continuous exposure. Secondly, because of concerns that toxicity may not be simply related to exposure concentration or tissue concentration during intermittent exposure, a range of sub-lethal end points relating to main physiological processes affected by Cd were examined. These included osmoregulation (tissue and plasma electrolytes, osmotic pressure), oxidative stress parameters (lysosomal membrane damage via neutral red retention, total glutathione,

thiobarbituric acid reactive substances or TBARS), as well as organ pathologies and haematology in order to overview animal health status.

3.1. Methodology

3.1.1. Test Organism

Mussels were collected in September, 2010 from Port Quin and acclimated as described (Section 2.2). The stock filtered seawater (mean ± standard error of the mean (SEM), n = 14) was tested daily for pH (pH 301 meter, Hanna Instruments, Leighton Buzzard, UK; 7.80 ± 0.02), salinity (YSI 63, refractometer, Fleet, UK; 34.60 ± 0.03 ppt), dissolved oxygen (Hach Lange LDO-HQ 10 DO meter, Salford, UK; 9.50 ± 0.13 mg l^{-1}) and total ammonia (HI 95715, Hanna Instruments; 1.04 ± 0.03 mg l^{-1}) and the water was renewed twice a week. Stock mussels were maintained as described (Section 2.2).

3.1.2. Experimental Design

One hundred and ninety-eight (198) mussels (whole weight, 9.5 ± 0.1 g; shell length, 43.2 ± 119 0.5 mm; means ± SEM) were randomly allocated into nine experimental glass aquaria containing 20 l of filtered seawater (Section 2.2). Each glass test vessel contained 22 mussels. The animals were not fed for 24 h prior to transfer to the test vessel, or during the experiment in order to minimise the risk of the Cd adsorption to food or faecal material, and to help maintain the water quality.

Two mussels/tank (a total of 18 mussels) were collected at day 0 (initial mussels). Six of these were analysed for Cd accumulation and haematology, and an additional six mussels were used for histology and biochemistry. The remaining

mussels in each tank (20 mussels/test vessel) were exposed using a semi-static exposure regime with 100% water change every 24 h, to either a control (filtered seawater only, no added Cd) or 50 µg Cd l^{-1} as $CdCl_2$ using both intermittent and continuous regimes (3 tanks/treatment; total of 60 mussels/treatment). Dosing was achieved by adding 1 ml of 1g Cd l^{-1} as $CdCl_2$ stock solution to treatment tanks (containing 20 l of seawater) to give a nominal concentration of 50 µg Cd l^{-1}. Mussels in the continuous regime tanks were exposed daily to Cd immediately after the daily water change, while the mussels in the intermittent regime tanks were exposed to Cd and returned to clean seawater alternately every two days. Because the focus of the research was on sub-lethal effect, the concentration of 50 µg Cd l^{-1} (as $CdCl_2$) was selected based on known sub-lethal concentration to *M. edulis* where Cd accumulation in the tissues and biological responses could be easily measured without mortality (Sheir and Handy, 2010). Seawater quality (as above) was checked and there were no significant differences between the tanks (ANOVA, $p > 0.05$). Overall values were (means ± SEM, $n = 126$ samples) pH, 7.9 ± 0.1; salinity, 34.0 ± 0.1 ppt; dissolved oxygen, 10.1 ± 0.1 mg l^{-1}; and total ammonia, 0.26 ± 0.1 mg l^{-1}. Water samples were also collected daily immediately before and after renewal of the test media. Background Cd concentration in the control filtered seawater ($n = 42$) was < 1 µg l^{-1}. On sampling days (at 2, 4, 6, 8, 10, 12, and 14 days), Two mussels per tank (6 mussels/treatment) were randomly sampled for tissue trace element analysis, plasma ions and osmotic pressure. For neutral red retention, haemocyte counts, plasma glucose and tissue biochemistry (total glutathione, TBARS) additional mussels (2 from each tank, 6 mussels/treatment) were sampled on days 6 and 14.

3.1.3. Heamolymph Extraction and Tissue Collection

Mussels were collected from tanks every 2 days and were first rinsed in clean filtered seawater to remove excess Cd, and haemolymph was collected as described (Section 2.4).

3.1.4. Trace Metal Analysis

Tissues Cd concentration and trace element composition were analysed according to the method described by Sheir and Handy (2010) as described in Chapter 2 of this thesis. All seawater samples were analysed for Cd by ICP-MS with a detection limit of 0.026 ± 0.1 µg Cd l^{-1}. The procedural detection limit for Cd analysis in tissue digests on the ICP-MS and ICP-OES were 0.2 and 3.7 Cd µg l^{-1} respectively and was derived from three times the standard deviation of the procedural blank. For a typical 0.1g of tissue the detection limit equates to 0.01 and 0.19 µg Cd g^{-1} dry weight tissues respectively.

3.1.5. TBARS and Total Glutathione Concentration

Tissues TBARS and total glutathione concentrations were determined as described (Section 2.7.1)

3.1.6. Neutral Red Retention and Total Haemocyte Count

The effect of Hg on the lysosomal integrity of the haemocytes was assessed by the neutral red retention (NRR) assay based on the ability of viable cells to incorporate

and bind the neutral red dye in the lysosomes. Haemocyte count was also performed (Sections 2.9 and 2.10).

3.1.7. Plasma Ion, Osmotic Pressure and Glucose Assay

To investigate the effect of Cd on osmoregulation, Na^+, K^+ and osmotic pressure were measured in the plasma (Section 2.11). Plasma glucose was measured based on the glucose oxidase method with slight modifications for microplate (Section 2.12).

3.1.8. Histological Examinations

Histological investigations were conducted as described (Section 2.6).

3.1.9. Statistical Analysis

Statistical analyses were done on all data by StatGraphics Plus for windows version 5.1 as described (Section 2.13).

3.2. Results

3.2.1. Aqueous Exposure to Cd

The intermittent and continuous exposure profiles were confirmed by the measured Cd concentrations in the tanks. Background Cd concentration in the control filtered seawater (n = 42, Figure 7) was < 1 µg Cd l^{-1}. The nominal 50 µg Cd l^{-1} exposure was confirmed by total concentration in the tanks and were (means ± SEM, µg l^{-1}) 55.9 ± 0.8 (n = 42) and 56.2 ± 1.1 (n = 21) for continuous and intermittent exposure respectively. There were no recorded mortalities of *M. galloprovincialis* in either the continuous or intermittent exposures as a result of the aqueous exposure to 50 µg Cd l^{-1} as $CdCl_2$, confirming the sub-lethal exposure.

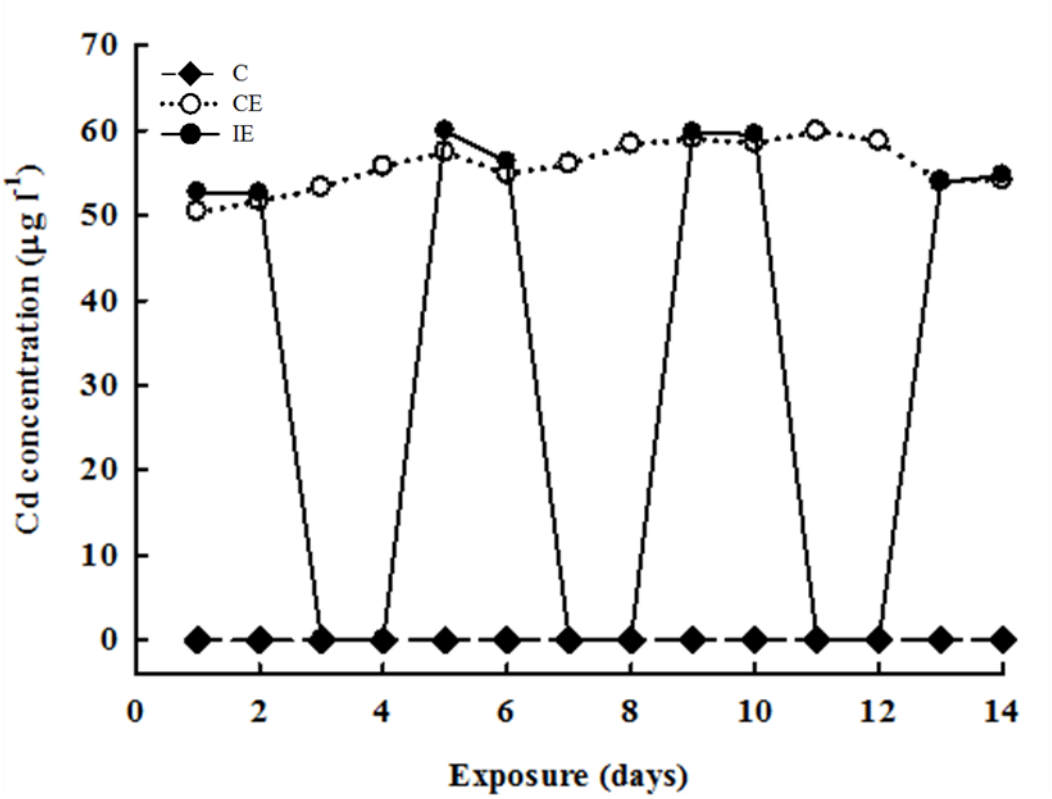


Figure 7: The cadmium concentration in seawater after 14 days exposure to control, C (no added Cd, diamonds on dashed line) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous, CE (open circles with a dotted line) or intermittent, IE (closed circles with a solid line) exposure. Water samples were collected at the beginning of each exposure day, immediately after the renewal of the test media. Measurements of Cd concentration just before renewal of the test media are not included for clarity. Data are means, µg l^{-1} for *n* = 3 tests per treatment at each exposure day. The detection limit was used to replace all values below detection limit. Error bars are not shown for clarity. The line on the intermittent exposure was drawn to emphasise the intermittent phases, it does not imply a gradual change in exposure concentration between dosing within each pulse, as full water changes were done manually in each tank.

3.2.2. Tissue Cd Accumulation

Tissue Cd concentrations in the control group were low and did not change over time, with values around 1-2 µg Cd g^{-1} dry weight or much less (Figure 8). The values for Cd accumulation of control animals showed tissue-specific differences, with the highest concentrations in the digestive gland and lower concentrations in gills followed by the remaining soft tissue, gonad and the posterior adductor muscle in rank order (Figure 8). Cd exposure to either continuous or intermittent exposures resulted in increased tissue concentration compared to the controls (ANOVA or Kruskal-Wallis, $p < 0.05$), from day 2 up to the end of the experiment. For example, at the end of the experiment, the Cd concentration in the digestive gland (means ± SEM, n = 4-6, µg Cd g^{-1} dry weight) was 2.1 ± 0.4, 85.7 ± 7.8, 58.5 ± 8.3 for control, continuous and intermittent exposure respectively.

In the continuous exposure mode, a two phase pattern of Cd accumulation was observed in most tissues examined (digestive gland, gill, gonad and remaining soft tissue, Figure 8). An initial uptake phase (up to day 6) followed by a more steady state phase (plateau) was observed during the rest of the experiment. The haemolymph showed an initial Cd uptake on day 2; thereafter a no statistically significant difference was observed in Cd accumulation over time up to day 10.

Mussels exposed to the intermittent exposure generally showed less Cd accumulation than the continuous profile (Figure 8). At the end of the experiment, the tissue Cd concentration in the intermittent exposure was significantly lower (≤ 2 folds) in the digestive gland, gill, remaining soft tissue (ANOVA, $p < 0.05$) and haemolymph (Kruskal-Wallis $p < 0.05$), compared to the continuous exposure mode. However, there was no significant difference between the continuous and the intermittent exposure for tissue Cd concentration in the adductor muscle and gonad at the end of the experiment (ANOVA, $p > 0.05$, Figure 8). Notably, the haemolymph and gonad showed an

alternating temporal pattern of uptake and clearance corresponding with the exposure phases of the intermittent profile (Figure 8). Like the continuous exposure counterpart, the digestive gland and gill of the intermittent exposure mode showed a two phase pattern of accumulation over time. An initial Cd uptake phase (up to day 6) and a steady state (tissue concentrations plateauing) up to the end of the experiment. For the posterior adductor muscle and the remaining soft tissue, there were no statistically significant differences in Cd accumulation after the initial uptake (day 2) up to the end of the experiment (Figure 8).

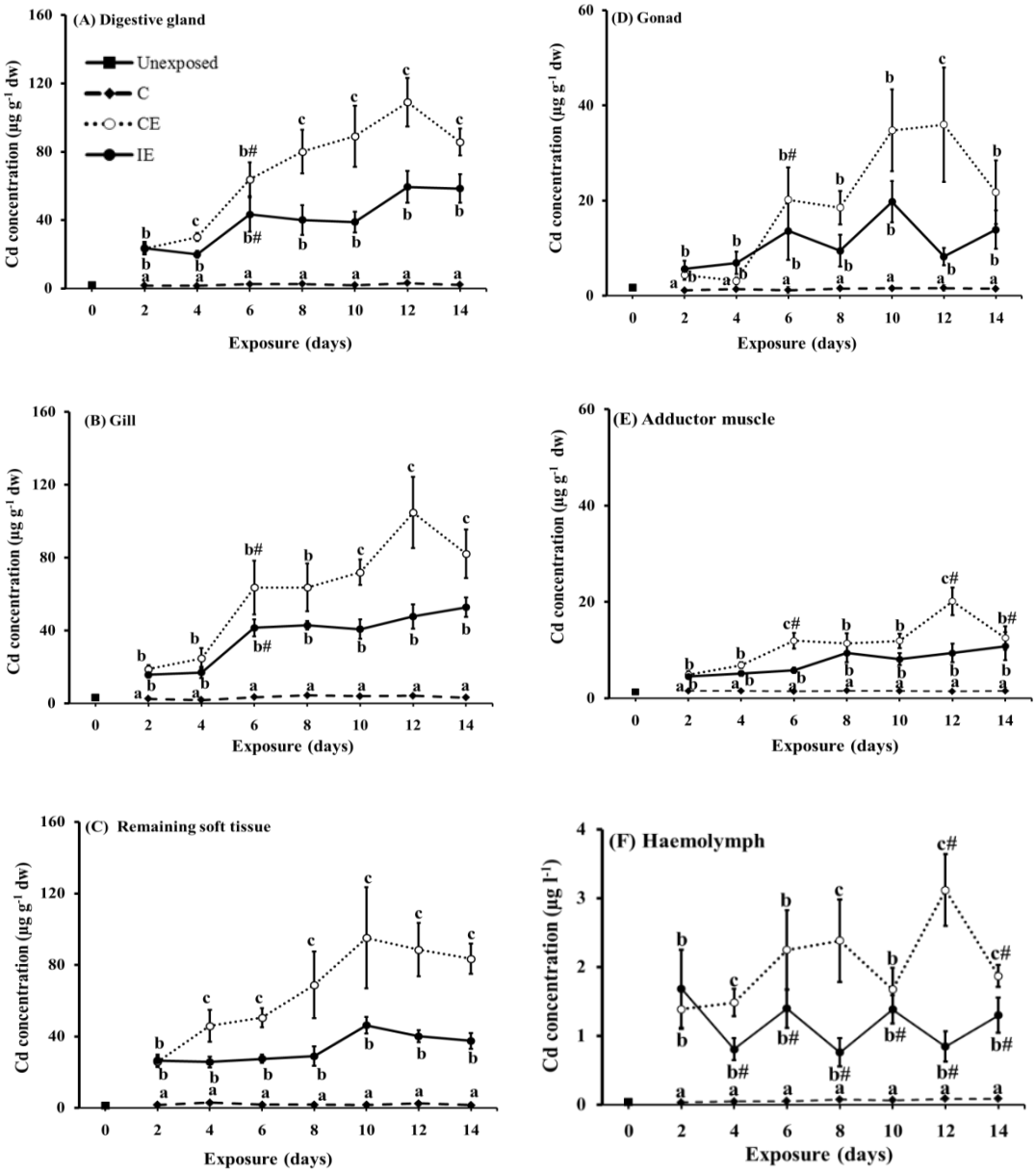


Figure 8: Cadmium concentrations in the (A) digestive gland, (B) gill , (C) remaining soft tissue, (D) gonad (E) posterior adductor muscle and (F) haemolymph after 14 days exposure to control, C (no added Cd, diamonds on dash line) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous, CE (open circles, dotted lines) or intermittent, IE (closed circles, solid lines) exposure. The black square at time zero represents the background Cd concentration in unexposed (initial) mussels at the start of the experiment. Data are means ± SEM, µg Cd g^{-1} dry weight tissue (dw), n = 4-6 mussels per treatment at each exposure day. Note the units for haemolymph are µg l^{-1}. Different letters within the exposure day indicates a significant treatment effect (ANOVA or Kruskal-Wallis, $p < 0.05$). # indicates a significant time effect within treatment compared to the previous exposure day (ANOVA or Kruskal-Wallis, $p < 0.05$). All Cd treatments, but not the control, showed a statistically significant difference compared to the initial mussel at time zero (labels not added for clarity). All analysis was done with ANOVA except the haemolymph.

3.2.3. Effects of Cd Exposure on Haemolymph Chemistry and Ion Regulation

Total haemocytes counts, neutral red retention by the haemocytes and solutes concentration (glucose concentration, osmotic pressure, Na^+ and K^+) in the cell-free haemolymph were performed at the start, middle and the end of the experiment (Table 7). There was no overall treatment-dependent effect on the total haemocytes counts for either Cd treatment compared to controls, but the continuous exposure was significantly decreased compared to the intermittent exposure at the end of the experiment (Kruskal-Wallis, $p = 0.006$, Table 7). The neutral red retention ability of the haemocytes did not show treatment dependent significant differences for all treatment including the control at the end of the experiment. However, there was a transient significant decrease on day 6 in the intermittent exposure compared to either the continuous exposure or control (Kruskal-Wallis, $p = 0.003$, Table 7). There was no overall Cd treatment effect on the glucose concentration in the cell-free hemolymph. However, there was a statistically significant transient decrease in glucose concentration in both Cd treatment compared to the control on day 6 (ANOVA, $p = 0.007$) but this was lost by the end of the experiment (ANOVA, $p = 0.43$, Table 7), and glucose concentrations remained low (< 1 mmol l^{-1}) in all treatments including the controls.

Some transient changes were observed in the haemolymph for osmotic pressure, Na^+ and K^+ concentrations over time within treatments during the experiment (Table 7). However, there were no Cd-dependent effects with values remaining in the normal range for marine mussels (e.g *M. edulis*) at the end of the experiment; 981-1051 mosmol kg^{-1}, 412-500 mmol l^{-1} and 8-13 mmol l^{-1} for osmotic pressure (ANOVA, $p > 0.05$), Na^+ and K^+ (Kruskal-Wallis, $p > 0.05$) respectively. Major electrolytes (Na^+, K^+, Mg^{2+} and Ca^{2+}) were also analysed in the digestive gland, gill, remaining soft tissue, gonad, and adductor muscle. There were no overall effects in all tissues for Na^+, K^+, Mg^{2+} and Ca^{2+} (Tables 8 to 11). However, there were some transient significant

changes for tissue electrolytes at some time points which were lost at the end of the experiment in the Cd treated groups. There was no statistically significant relationship between tissue Cd and Ca^{2+} levels in digestive glands, gill, remaining soft tissue, gonad, or adductor muscle with Pearson linear correlation analysis. Values were ($p > 0.05$, $r =$ 0.14; -0.20 ; -0.12; 0.02; 0.02; 0.02) for digestive glands, gill, remaining soft tissue, gonad, or adductor muscle respectively.

Table 7: Total haemocyte counts (THC), neutral red retention (NRR) and solute concentration in the cell-free haemolymph from *M.galloprovincialis* after 14 days exposure to 0 (no added Cd control) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous or intermittent exposures

Parameters	Treatments	Exposure (Days)							
		0	2	4	6	8	10	12	14
THC	Control	1.24 ± 0.20			1.61 ± 0.1a				1.26 ± 0.2ab
(*10^6cells ml^{-1})	Continuous	-			2.02 ± 0.2a+				0.84 ± 0.1a#
	Intermittent	-			1.52 ± 0.2b+				2.62 ± 0.8b+
NRR (OD	Control	6.70 ± 0.38			4.35 ± 0.60a+				8.03 ± 1.13#
10^6cells^{-1})	Continuous	-			4.60 ± 0.46a+				11.87 ± 2.20 #+
	Intermittent	-			0.32 ± 0.03b+				7.50 ± 1.80 #
Glucose	Control	0.55 ± 0.01			0.55 ± 0.01a				0.57 ± 0.01
(mmol l^{-1})	Continuous	-			0.52 ± 0.01b				0.54 ± 0.01
	Intermittent	-			0.52 ± 0.01b				0.54 ± 0.02

Continuation of Table 7: Total haemocyte counts (THC), neutral red retention (NRR) and solute concentration in the cell-free haemolymph

Parameters	Treatments	Exposure (Days)							
		0	2	4	6	8	10	12	14
Osmotic pressure (mosmol kg^{-1})	Control	1051.2 ± 2.1	1026 ± 3.3a+	1033.8 ± 1.3a+	1016.2 ± 5.4#+	999 ± 2.8#+	994.8 ± 1.4+	979.2 ± 1#+	1005.8 ± 3.3#+
	Continuous	-	1032.6 ± 4.3a+	1021 ± 4.9b+	1025.2 ± 2.9+	1002.5 ± 1.5+	997.2 ± 0.6+	981.8 ± 2.7#+	1006.7 ± 6.6 #+
	Intermittent	-	1043.2 ± 3.6b	1020.8 ± 3.8b+	1024.8 ± 7.7+	1004.6 ± 2.8+	990.8 ± 3.6+	985.6 ± 1.5#+	1007 ± 3.1#+
Na^+ (mmol l^{-1})	Control	458.7 ± 3.4	461.9 ± 4.8a	421.5 ± 5.1a#+	432.5 ± 2.7#+	444.5 ± 11.0	500.6 ± 20.4	447.6 ± 5.2#	432 ± 8.6+
	Continuous	-	436.3 ± 5.5b+	412.2 ± 6.5b#+	481.8 ± 33.7#	428.9 ± 17.7+	485 ± 6.1#+	425.5 ± 9.7#+	431.4 ± 8.4+
	Intermittent	-	421.9 ± 5.5b+	438 ± 2.2b#+	421.2 ± 13.6+	447.7 ± 8.8#	475 ± 13.4#+	440.3 ± 7.0#	414.7 ± 2.5#+
K^+ (mmol l^{-1})	Control	11.5 ± 1.2	10.9 ± 0.4	8.8 ± 0.2a#+	8.5 ± 0.3+	12.9 ± 2.9	10.8 ± 0.2a	10.3 ± 0.4	9.0 ± 1.4+
	Continuous	-	10.2 ± 0.5	9.9 ± 0.2b+	9.6 ± 1.0	12.9 ± 0.6#	10.6 ± 0.5b#+	10.4 ± 0.6	8.9 ± 0.3+
	Intermittent	-	9.8 ± 0.2+	10 ± 0.2b+	11.5 ± 1.3	15.5 ± 3.1	11 ± 0.2b#	10.7 ± 0.3	7.9 ± 0.2#+

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA or Kruskall–Wallis, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA or Kruskall-Wallis, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA or Kruskall–Wallis, $p < 0.05$). Note, only the glucose and osmotic pressure data were parametric and analysed using ANOVA, all other data were analysed by the Kruskall–Wallis test.

Table 8: Na^+ concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days exposure to 0 (no added Cd control) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous or intermittent exposures.

Tissues	Treatments	Exposure days							
		0	2	4	6	8	10	12	14
Digestive gland	Control	289.5 ± 12.9	252.7 ± 22.0+	267.1 ± 43.8#	306.9 ± 6.2	292.2 ± 17.1	220.6 ± 18.7+	364.1 ± 62.9	217.1 ± 22.4+
	Continuous		246.2 ± 17.1	221.5 ± 19.3	296.8 ±11.2	238.5 ± 39.6	246.3 ± 18.2	226.7 ± 20.9	281.3 ± 33.8
	Intermittent		271.3 ± 24.3	295.3 ± 29.0	336.1 ± 42.5	259.8 ± 17.1	234.0 ± 34.8	277.2 ± 35.2	239.7 ± 31.9
Gill	Control	622.9 ± 40.5	662.7 ± 26.5	575.8 ± 69.9	789.6 ± 15.4a#+	746.2 ± 32.7+	658.9 ± 16.7+	663.2 ± 90.9+	541.3 ± 36.5#+
	Continuous		643.0 ± 54.1	680.4 ± 18.7	807.2 ± 35.5a+	781.1 ± 39.6+	659.9 ± 46.6	595.6 ± 50.7	546.2 ± 26.7
	Intermittent		735.0 ± 42.4+	637.6 ± 24.6	645.7 ± 34.5b	698.1 20.8a	601.8 ± 31.0	533.4 ± 17.6	537.8 ± 53.7
Remaining soft tissue	Control	383.3 ± 33.1	361.4 ± 22.4	425.8 ± 33.8	436.6 ± 11.6	391.6 ± 19.4	344.1 ± 27.5	414.3 ± 52.9	291.0 ± 25.8#+
	Continuous		396.7 ± 15.4	356.9 ± 16.7	449.6 ± 17.4#	343.8 ± 31.3#	352.4 ± 25.1	332.2 ± 14.4	360.1 ± 23.2
	Intermittent		407.0 ± 25.8	409.8 ± 35.2	400.1 ± 26.5	342.8 ± 20.0	366.4 ± 46.5	358.7 ± 16.7	311.0 ± 26.2
Gonad	Control	450.0 ± 183.8	327.3 ± 49.6	470.8 ± 33.8	452.7 ± 41.0	490.3 ±92.4	241.1 ± 40.9	522.0 ± 120.3	260.0 ± 91.6
	Continuous		379.8 ± 62.2	219.0 ± 30.0	437.6 ± 85.4	222.8 ± 40.3	291.1 ± 28.4	332.5 ± 68.1	283.7 ±21.9
	Intermittent		372.5 ± 88.0	466.4 ± 123.5	455.7 ± 127.4	335.6 ± 52.2	356.6 ± 82.9	360.2 ± 49.8	253.1 ± 53.6
Adductor muscle	Control	303.2 ± 32.3	262.2 ± 16.2a	241.2 ± 31.6	274.2 ± 17.8	386.5 ± 50.6#	257.8 ± 11.5#	288.2 ± 9.3a#	203.1 ± 15.6#+
	Continuous		345.1 ± 20.9b	274.4 ± 20.7	299.9 ± 18.9	294.0 ± 31.2	290.7 ± 28.3	252.6 ± 13.8a	240.2 ± 4.1
	Intermittent		300.1 ± 11.4a	269.5 ± 26.3	270.1 ± 17.5	331.0 ± 34.6	238.1 ± 29.2	232.1 ± 15.9b	210.4 ±18.2

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA or Kruskall-Wallis, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA or Kruskall-Wallis, $p < 0.05$).
+ Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA or Kruskall-Wallis, $p < 0.05$).

Table 9: Ca^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days exposure to 0 (no added Cd control) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous or intermittent exposures.

Tissue	Treatments	Exposure (days)							
		0	2	4	6	8	10	12	14
Digestive gland	Control	41.3 ± 4.8	24.3 ± 2.2+	22.1 ± 2.7#+	27.0 ± 3.2+	24.8 ± 2.5a+	17.8 ± 1.6+	35.7 ± 7.7#	28.8 ± 3.5#+
	Continuous		20.9 ± 1.8+	23.7 ± 4.2+	23.1 ± 0.7+	17.3 ± 0.5b#+	21.6 ± 1.1#+	22.3 ± 0.9+	27.5 ± 2.3+
	Intermittent		24.9 ±3.6	22.2 ± 2.6	26.1 ± 2.7	23.4 ± 0.9a	21.2 ± 2.2	54.9 ± 21.4	42.2 ± 11.8
Gill	Control	42.5 ± 2.2	45.5 ± 1.4	37.4 ± 5.4	47.4 ± 2.3#+	50.3 ± 1.6+	38.8 ± 2.0#+	49.9 ± 6.2#+	40.5 ± 1.9#
	Continuous		51.6 ± 4.5	42.7 ±1.8	51.1 ± 3.7	48.5 ± 7.8	41.8 ± 2.8	42.5 ± 3.5	40.4 ± 3.1
	Intermittent		46.5 ± 2.7	39.2 ± 2.3	44.2 ± 2.4	42.4 ± 0.3	42.1 ± 4.4	39.3 ± 1.4	40.5 ± 3.6
Remaining soft tissue	Control	109. 3 ± 11.6	110. 6 ± 19.7	74.3 ± 6.1	112.6 ± 47.1	46.2 ± 5.0+	45.4 ±10.2+	65.4 ± 10.2	49.3 ± 7.1+
	Continuous		98. 4 ± 13.7	66.6 ± 8.6+	53.2 ± 4.9#+	49.5 ± 9.8+	72.9 ± 14.7+	38.8 ± 4.8#+	59.6 ± 11.4+
	Intermittent		107.0 ± 19.5	44.5 ± 3.8#+	63.1 ± 9.7	34.9 ± 1.3	72.0 ± 17.6	42.4 ± 4.2+	38.1 ± 4.2+
Gonad	Control	43.6 ± 16.2	20.9 ± 3.0	27.0 ± 5.3	31.4 ± 5.2	36.7 ± 5.8a	14.7 ± 1.9#+	38.4 ± 7.7#	20.6 ± 5.2#
	Continuous		27.0 ± 4.7	18.4 ± 3.7	28.1 ± 4.9	16.0 ± 1.7b	19.1 ± 1.8	23.1 ± 4.0	20.7 ± 2.4
	Intermittent		30.4 ± 8.5	31.5 ± 10.8	23.5 ± 6.0	17.9 ± 0.7b	34.9 ± 12.1	22.8 ± 1.9	18.0 ± 1.1
Adductor muscle	Control	46.5 ± 11.9	46.4 ± 4.3	30.0 ± 7.4	62.7 ± 19.3	53.2 ± 7.0	31.7 ± 6.3	45. 7 ± 2.8	30.5 ± 4.9
	Continuous		44.1 ± 2.2	36.7 ± 5.9	44.7 ± 9.4	39.6 ± 4.5	29.5 ± 5.2	66.9 ± 21.2	30.4 ± 1.2
	Intermittent		49.4 ± 7.8	27.0 ± 5.7	40.4 ± 4.2	44.6 ± 8.5	32.1 ± 5.5	45.5 ± 11.1	39.0 ± 6.6

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA or Kruskall-Wallis, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA or Kruskall-Wallis, $p < 0.05$).
+ Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA or Kruskall-Wallis, $p < 0.05$).

Table 10: K^+ concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days exposure to 0 (no added Cd control) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous or intermittent exposures

Tissue	Treatments	Exposure (days)							
		0	2	4	6	8	10	12	14
Digestive gland	Control	205.1 ± 4.3	198.4 ± 8.6	267.1 ± 24.8	219.4 ± 6.5	217.6 ± 13.3a	189.2 ± 11.6#	240.2 ± 12.3a#+	167.3 ± 7.3#+
	Continuous		203.4 ± 6.4	187.1 ± 6.6	227.9 ± 8.9	198.7 ± 16.7	197.6 ± 6.7	203.8 ±3 9.5b	191.5 ± 7.9
	Intermittent		206.3 ± 7.3	207.4 ± 10.9	220.5 ± 15.4	202.0 ± 10.7	180.3 ± 18.4	187.3 ± 5.9b	184.3 ± 10.1
Gill	Control	253.2 ± 3.2	261.3 ± 5.5	249.8 ± 4.2	281.0 ± 7.1a#+	294.1 ± 7.3+	264.7 ± 11.7a#	267.0 ± 13.3 a#+	219.9 ± 3.1a#+
	Continuous		277.7 ± 10.1+	248.6 ± 5.6	277.6 ± 8.9a#+	307.6 ± 11.3+	259.4 ± 15.6a#	203.8 ± 9.5b#	213.8 ± 1.7a#+
	Intermittent		274.6 ± 9.9+	253.1 ± 5.5#	252.2 ± 6.1b	276.9 ± 6.9#+	210.0 ± 4.4b#+	235.0 ± 1.5c+	205.5 ± 6.5b#+
Remaining soft tissue	Control	214.8 ± 4.0	213.9 ± 6.9	232.4 ± 5.1a	27.7 ± 3.9	228.8 ± 6.8a	197.7 ± 5.2#+	228.9 ± 10.3#	181.9 ± 6.4 a#+
	Continuous		226.7 ± 8.6	211.4 ± 3.3b	226.3 ± 5.0	203.2 ± 8.1b#	203.0 ± 3.6	214.7 ± 2.1	204.9 ± 6.3b
	Intermittent		214.6 ± 6.0	214.9 ± 5.6b	218.5 ± 4.9	198.6 ± 5.3b#	192.6 ± 7.2+	213.7 ±7.5#	179.8 ±4.6a#+
Gonad	Control	205.0 ± 5.1	223.3 ± 12.3a	219.3 ± 12.3	255.4 ± 16.9+	220.2 ± 11.9a	184.9 ± 14.9	236.8 ± 16.8	156.8 ± 17.0#+
	Continuous		216.4 ± 11.0b	198.1 ± 11.2	258.9 ± 14.4#+	168.1 ± 10.8b+	211.0 ± 11.2#	218.3 ± 14.6+	197.5 ± 5.4
	Intermittent		237.8 ± 18.9b	207.6 ± 9.9	216.6 ± 17.2	208.8 ± 8.9a	191.5 ± 16.3	197.9 ± 14.2	169.6 ± 11.2
Adductor muscle	Control	189.8 ± 2.9	223.0 ± 5.5+	204.1 ± 4.9	225.0 ± 4.0#+	278.8 ± 30.3a#+	207.3 ± 3.1#+	218.4 ± 6.2+	160.0 ± 2.3#+
	Continuous		215.2 ± 5.8+	200.9 ± 3.6	218.3 ± 5.1+	221.5 ± 6.2a#+	203.6 ± 7.6	195.0 ± 9.3	173.1 ± 5.8#+
	Intermittent		208.3 ± 8.1	202.4 ± 5.0	217.5 ± 8.7	225.1 ± 6.1b+	162.2 ± 6.8#+	189.1 ± 8.3#	171.1 ± 5.0

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA or Kruskall-Wallis, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA or Kruskall-Wallis, $p < 0.05$).
+ Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA or Kruskall-Wallis, $p < 0.05$).

Table 11: Mg^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days exposure to 0 (no added Cd control) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous or intermittent exposures

Tissue	Treatments	Exposure (days)							
		0	2	4	6	8	10	12	14
Digestive gland	Control	79.1 ± 3.2	71.7 ± 5.5+	74.4 ± 9.9	83.8 ± 1.1	83.0 ± 5.0	64.7 ± 5.0+	100.2 ± 15.6	61.8 ± 5.8+
	Continuous		72.9 ± 3.9	63.8 ± 4.7	83.0 ± 2.3	69.7 ± 9.7	69.4 ± 4.0	68.0 ± 5.3	78.5 ± 7.8
	Intermittent		78.5 ± 4.8	80.3 ± 6.7	89.6 ± 9.7	75.3 ± 8.1	64.1 ± 7.2	80.0 ± 10.7	67.4 ± 7.3
Gill	Control	155.7 ± 8.9	169.5 ± 5.6	162.0 ± 9.6	198.0 ± 3.4a#+	193.4 ± 6.3+	167.8 ± 3.1#	190.2 ± 7.6a+	139.5 ± 7.8#
	Continuous		168.1 ± 11.3	171.0 ± 3.7	202.8 ± 7.6a#+	202.5 ± 8.2+	166.3 ± 10.6#	155.6 ± 10.9b	141.3 ± 6.5
	Intermittent		183.9 ± 8.7+	162.5 ± 5.0	164.3 ± 6.9b	180.0 ±4.0+	150.5 ± 6.7#	141.8 ± 3.6b+	139.9 ± 11.2
Remaining soft tissue	Control	104.9 ± 7.8	103.2 ± 6.8	114.3 ± 7.6	118.7 ± 2.3	108.7 ± 4.0	96.7 ± 6.1	115.6 ± 11.6	85.6 ± 6.6
	Continuous		109.3 ± 3.5	98.9 ± 4.2	119.8 ± 3.7#	96.6 ± 7.4	96.7 ± 5.6	97.7 ± 3.4	102.6 ± 5.1
	Intermittent		111.8 ± 6.1	112.1 ± 9.0	112.1 ± 8.5	97.8 ± 5.1	99.0 ± 8.9	102.6 ± 4.1	88.6 ± 5.5
Gonad	Control	117.7 ± 43.9	87.1 ± 11.4	117.8 ± 23.9	117.8 ± 23.9	123.7 ± 20.3	67.3 ± 9.4	131.0 ± 12.0	70.2 ± 20.0
	Continuous		97.7 ± 11.5	63.3 ± 7.7	112.5 ± 17.4	92.6 ± 31.7	78.9 ± 6.5	90.9 ± 14.5	79.3 ± 5.0
	Intermittent		97.5 ± 19.1	118.0 ± 27.8	116.0 ± 29.1	89.4 ± 10.4	89.7 ± 17.4	96.2 ± 10.9	69.1 ± 12.3
Adductor muscle	Control	81.6 ± 7.3	75.1 ± 3.6a	67.7 ± 8.4	79.0 ± 4.4	107.3 ± 12.2#	73.4 ± 2.4#	82.6 ± 2.4a#	59.9 ± 3.5#+
	Continuous		93.5 ± 4.6b	76.7 ± 4.5	85.2 ± 4.1	85.4 ± 7.0	80.0 ± 6.7	77.0 ± 5.5a	68.5 ± 1.1
	Intermittent		82.7 ± 2.5a	76.1 ± 5.9	75.1 ± 4.5	93.2 ± 8.1#	66.1 ± 6.2+	68.2 ± 3.3b	61.0 ± 4.2+

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA or Kruskall-Wallis, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA or Kruskall-Wallis, $p < 0.05$).
+ Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA or Kruskall-Wallis, $p < 0.05$).

3.2.4. Effect of Cd Exposure on TBARS Concentration

There were no clear treatment- or time-dependent changes in the TBARS concentrations of the gill, gonad or adductor muscle (ANOVA, $p > 0.05$, Figure 9). However, in the digestive gland the TBARS concentration of mussels from the continuous exposure showed a statistically significant decrease on day 6 onwards compared to the intermittent Cd treatment (ANOVA, $p = 0.04$, Figure 9). However, there were no differences between the controls compared to either the intermittent or continuous treatment. The Pearson correlation analysis showed no statistically significant correlations between Cd concentrations and TBARS in the digestive gland, gill, gonad and adductor muscle ($r = - 0.20$; $- 0.19$; $- 0.19$; $- 0.28$, $p > 0.05$) respectively.

3.2.5. Effect of Cd Exposure on Total Glutathione Concentration

Total glutathione concentrations were also measured in the tissue. There was no-overall treatment effect on total glutathione concentration in the digestive gland, gill, gonad or adductor muscle by the end of the experiment (ANOVA, $p > 0.05$, Figure 10). The Pearson linear correlation analysis showed no statistically significant relationship between Cd concentration and total glutathione levels in the digestive gland, gill, gonad or adductor muscle ($r = - 0.05$; 0.49; $- 0.64$ and 0.16, $p > 0.05$) respectively.

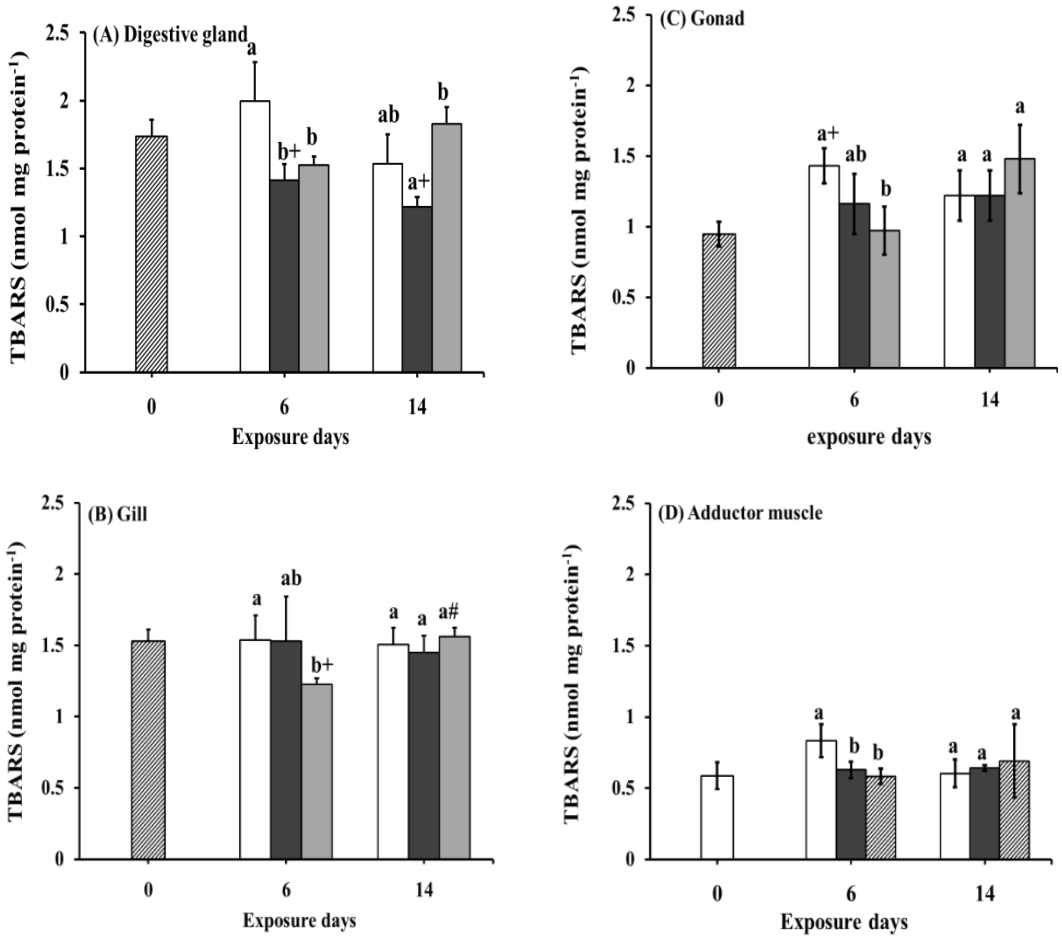


Figure 9: Thiobarbituric acid reactive substances (TBARS) concentration in whole tissue homogenates of the (A) digestive gland, (B) gill , (C) gonad, and (D) posterior adductor muscle after 14 days exposure to control (no added Cd, white bar) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous (black bar) or intermittent (grey bar) exposure. The hatched bar at time zero are values for unexposed (initial) mussels at the start of the experiment. Data are means ± SEM, nmol mg protein^{-1} for n = 4-6 mussels per treatment at each exposure day. Different letters within exposure day indicates statistically significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, stock mussel; ANOVA, $p < 0.05$).

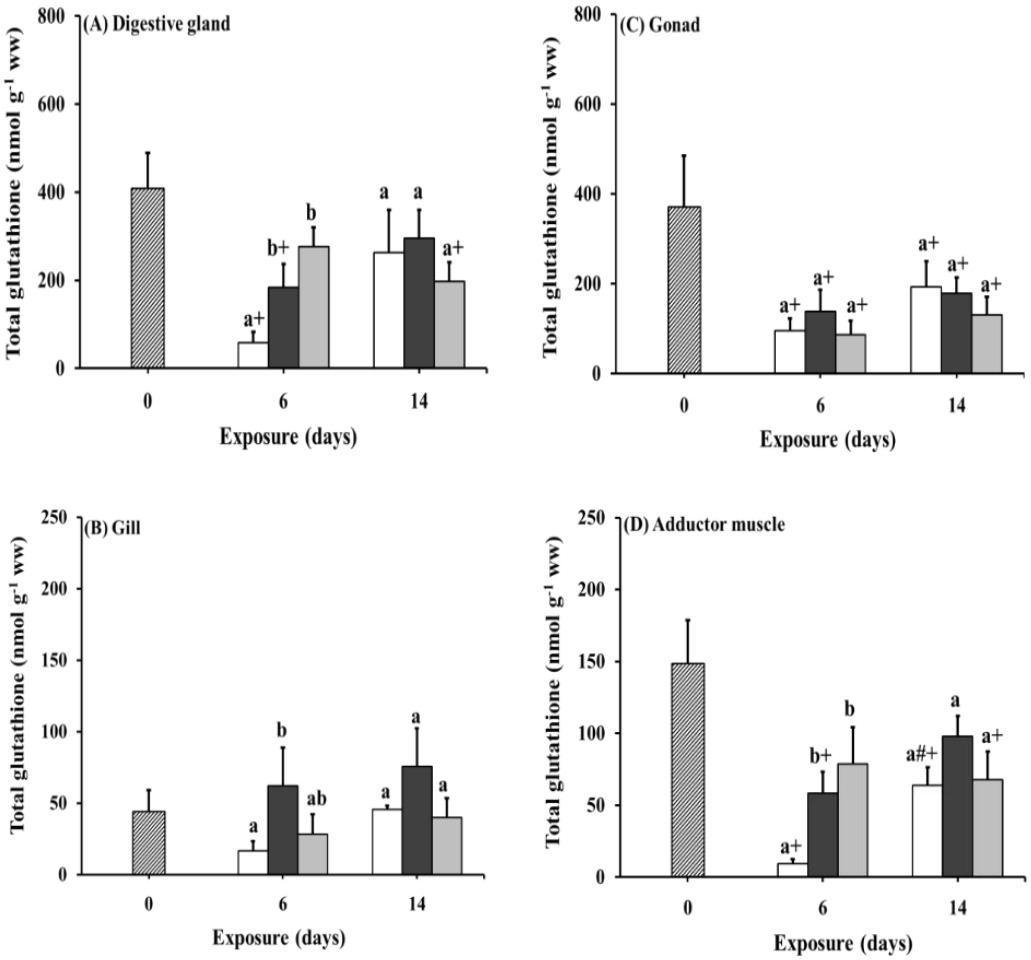


Figure 10: Total glutathione concentration in whole tissue homogenates of the (A) digestive gland, (B) gill , (C) gonad, and (D) posterior adductor muscle after 14 days exposure to control (no added Cd, white bar) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous (black bar) or intermittent (grey bar) exposure. The hatched bar at time zero are values for unexposed (initial) mussels at the start of the experiment. Data are means ± SEM, nmol g^{-1} wet weight (ww) of tissue for n = 4-6 mussels per treatment at each exposure day. Different letters within exposure day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, initial mussel stock) (ANOVA, $p < 0.05$).

3.2.6. Histological Alterations during Aqueous Exposure to Cd

The histology of the gill at the end of the experiment is shown (Figure 11A, B and C). The gill filaments from the control group showed normal lamellae, with the presence of frontal and lateral cilia, and no evidence of haemocyte infiltration in the long lacuna space or any other pathology (Figure 11A). There were no treatment-dependent differences in gill morphology, apart from an increase in the incidence of hyperplasia (increase in cell number) at the tips of the lamellae in both Cd treatments (Figure 11B and C).

The digestive gland was also examined (Figure 11D, E and F). The digestive gland of the control mussels exhibited normal architecture with well-defined digestive tubules and no evidence of pathology (Figure 11D). In contrast, there was some treatment dependent increase in the fractional area of the digestive gland occupied by haemocytes (i.e., an inflammatory haemocyte infiltration into the tissue) in both the Cd-exposed groups compared to the control. Three out of five mussels examined in either the continuous or intermittent exposure showed a clear haemocyte infiltration into the connective tissue (Figure. 11E and F). The % of the fractional area in the digestive gland occupied by haemocytes was (means ± SEM, $n = 5$) < 1 % (not observed), 40 ± 14, 20 ± 10 % for the control, continuous and intermittent treatments respectively (ANOVA, $p = 0.006$) at the end of the experiment. This occurred without changes in the dimensions of the digestive tubules. For example, the height (thickness) of the epithelial cells in the wall of the digestive tubules was (means ± SEM, $n = 5$) were 36 ± 3, 33 ± 5 and 37 ± 8 μm for control, continuous and intermittent exposure respectively (no statistically significant difference, ANOVA, $p > 0.05$).

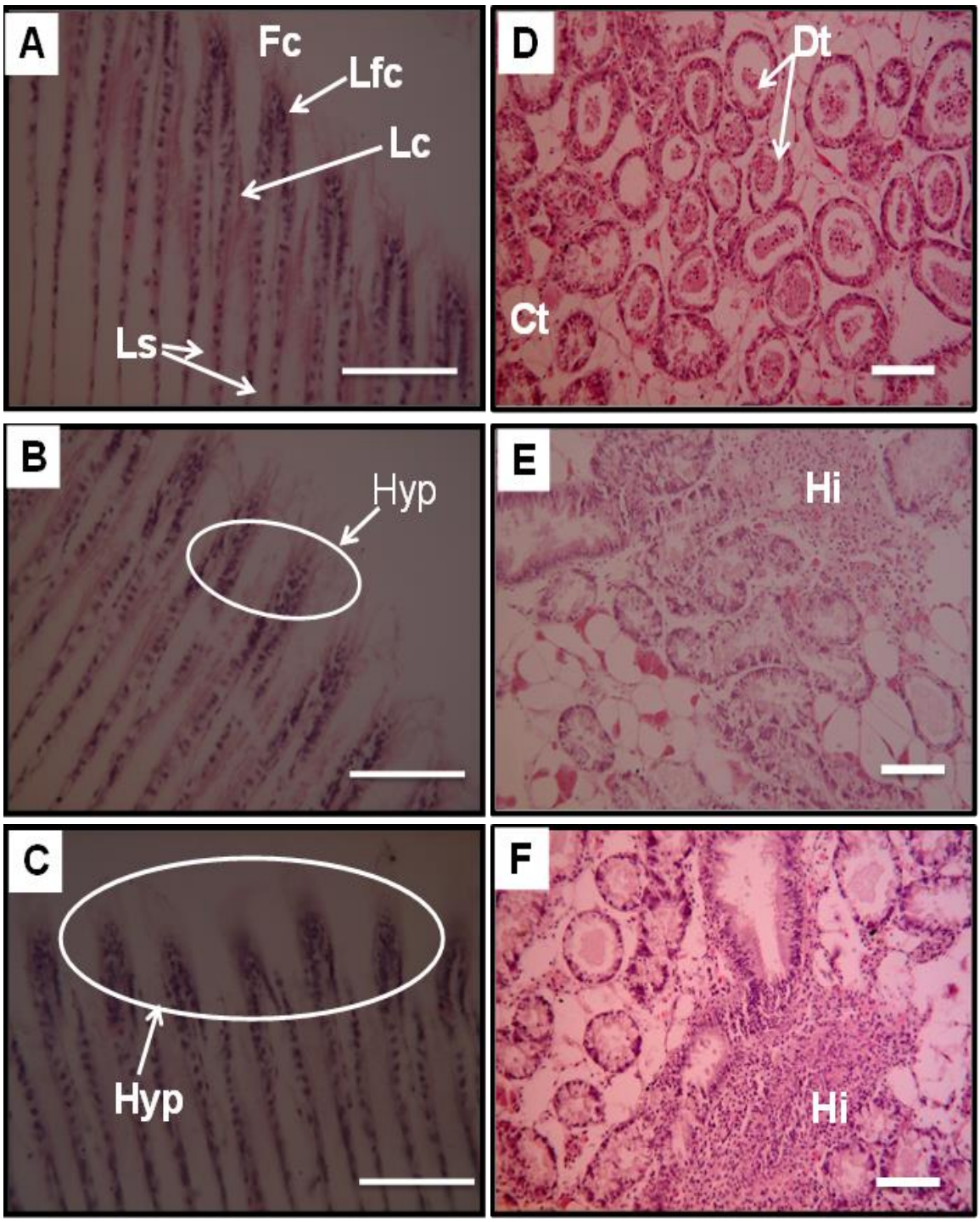


Figure 11: Histology of the gill (A-C) and digestive gland (D-F) of *M. galloprovincialis* after 14 days exposure to control (no added Cd, A, D) or 50 µg Cd l^{-1} as $CdCl_2$ in continuous (B, E) or intermittent (C, F) exposure: Slides were stained with Mayer's Haemotoxylin and Eosin. The circle represents gill filaments with hyperplasia (Hyp) on the tips. Fc, frontal cilia; Lfc, Laterofrontal cilia; Lc, Lateral cilia; Ls, Lacuna space; Ct, Connective tissues; Dt, Digestive tubules; Hi, Haemocyte infiltration. Scale bar: 50 µm, $n = 6$.

3.3. Discussion

The present study provides a detailed overview of the accumulation pattern and biological responses of *M. galloprovincialis* exposed to equal peak concentrations of Cd as $CdCl_2$ in continuous compared to intermittent exposure. To the best of our knowledge, this is the first study that has measured metal accumulation and sub-lethal responses in the same experiment with this species to compare continuous with intermittent exposure. Overall, the target organs for Cd accumulation remained the same in the two exposure regimes, but mussels from the intermittent Cd exposure accumulated generally less Cd than the continuous profile (Figure 8). Most internal organs did not clear Cd between the intermittent exposure pulses, but the haemolymph and gonad were dynamic showing increases and decreases in Cd concentrations that corresponded with the exposure profile. Despite differences in Cd tissue concentration between the continuous and the intermittent groups, the biological effects of the exposures were similar. Although there were some transient changes in some end points, by the end of the experiment there were generally no differences in haemolymph chemistry, tissue electrolytes, TBARS or glutathione between the Cd exposure regimes. However, there were treatment-dependent changes in haemocyte concentrations in the blood; and with an inflammatory haemocyte infiltration in the digestive gland.

3.3.1. Aqueous Exposure to Cd

The measured Cd concentrations in the seawater (Figure 7) confirmed that the intermittent exposure had similar peak Cd concentration to the continuous exposure, but in between pulses the concentrations of Cd, as expected, were low. Overall, the mussels exposed to the intermittent profile therefore received a total Cd dose about half that of the continuous exposure group. The absence of mortalities in the present study,

confirmed the expected sub-lethal exposure, similar to our previous findings with 50 µg Cd l^{-1} (Sheir and Handy, 2010).

3.3.2. Tissue Cd Accumulation

Both Cd treatments accumulated Cd in the internal organs (Figure 8) and showed a similar order of Cd distribution, with the highest concentrations in the digestive gland > gill > remaining soft tissue > gonad > adductor mussel. This result supports earlier findings in our laboratory, where Cd accumulation was also greatest in the digestive gland and gill, but lower in the adductor muscle after 11 days exposure to 50 µg Cd l^{-1} as $CdCl_2$ (Sheir and Handy, 2010).

The continuous exposure was characterised by a gradual rise in tissue Cd concentrations in the digestive gland and gills, which had begun to plateau from about day 10, and with haemolymph Cd concentrations remaining around 2-3 µg l^{-1} (Figure 8). The increasing trend of tissue Cd concentrations of the internal organs over time is consistent with the idea that unidirectional uptake rates for Cd, exceeds whole body clearance in *M. edulis* (Borchardt, 1983), leading to a gradual rise in Cd accumulation in the tissues. In contrast, the intermittent profile showed more step-wise increases of Cd concentrations in the digestive gland (Figure 8A), and clear increases/decreases in Cd concentration in the haemolymph with the exposure profile (Figure 8F). The gonad also showed evidence of intermittent accumulation with the exposure phase (Figure 8D). This phenomenon has been noted before for Hg exposures in fishes, where for example, whole body Hg increases only in the exposure phase of intermittent exposure in fathead minnows (Handy, 1995). This pattern of Cd accumulation is also broadly similar to previous reports on mussels re-exposed to Cd-containing water after some time to air (Coleman, 1980). The haemolymph Cd concentrations (Figure 8) confirmed the

intermittent profile was reflected inside the mussels, with the mussels showing some Cd clearance from the haemolymph when placed in clean water between Cd pulses. However, the Cd may not have been excreted as the Cd concentrations in other tissue compartments continued to rise in the clean water phase; including the digestive gland, gill and adductor muscle (Figure 8). This suggests the mussels were redistributing Cd from the haemolymph to the tissues during intermittent exposure.

Conventional theory on the dose-response relationship predicts that the intermittent exposure regime here should result in half the Cd accumulation of the continuous exposure regime; because in the former, mussels were exposed to Cd for half as long, with fifty percent of the time in clean water (Handy, 1994). However, the notion of calculating the sum of the exposure concentrations to estimate the likely exposure of the tissues only applies when the time in clean water is much shorter than the clearance rate of the animal for the substance (Handy, 1994). In such circumstances, the time in clean water is insufficient to allow excretion of the metal, and thus a steady (or step-wise additive) rise in tissue metal levels is observed. In this study, after day 4, this appeared to be the case for the digestive gland, gill and adductor muscle which presumably have longer clearance times (Borchardt, 1983), than the two days the mussels had in clean water between pulses of Cd. The haemolymph, in contrast, appeared to have a short clearance time for Cd and this was reflected in rapid dynamic changes in Cd concentration with the intermittent exposure profile (Figure 8). Data on Cd half times in the haemolymph of *M. galloprovincialis* appear to be lacking, but according to Bjerregaard (1990), shore crabs *Carcinus maenas*, injected with radiolabeled Cd, can remove half the Cd from the haemolymph in approximately 10 hours. The result of the present study (Figure 8F) shows that *M. galloprovincialis* can remove about half the Cd concentration from the haemolymph during the 48 hours in clean water, but equally, the Cd is restored quickly to the previous level on re-

commencing the Cd exposure. Thus, the dynamics of Cd accumulation is tissue-specific with "fast" tissues being sensitive to the intermittent exposure profile, while "slow" tissues (adductor muscle and remaining soft tissue) are not.

3.3.3. Effects of Cd Exposure on Haemolymph Chemistry and Ionic Regulation

There were no statistical differences in the total haemocyte counts (THC) in the haemolymph of mussels from either Cd treatment compared to controls (Table 7), although there was an exposure regime-effect with the THC being lowered in the continuous compared to the intermittent exposure. However, this difference was < 1 x 10^6 cells ml^{-1}, and well within the normal variability of haemocyte counts in *M. edulis* (Mayrand et al., 2005). Neutral red retention was also measured, and is regarded as a useful biomarker of the general health of the cells (Lowe et al., 1995). In the present study NRR was not affected by Cd exposure. This finding is consistent with previous studies (Sheir and Handy, 2010) which also reported no Cd-treatment effect on NRR or phagocytosis ability of *M. edulis* after 11 days continuous exposure to 50 µg Cd l^{-1} as $CdCl_2$. Together, these observations suggest that the circulating haemocytes were probably normal and largely unaffected by the mode of Cd exposure. There was no overall Cd treatment effect on glucose concentration in the present study (Table 7 all < 1 mmol l^{-1}) and the values were typical of unfed mussels held in the laboratory for a few days (Sadok et al., 1997), and the absence of elevation of haemolymph glucose suggests no acute stress response.

There were no major differences in cell-free haemolymph ion concentrations, or tissue electrolyte composition, apart from some transient K^+ depletion in the gill tissue of both Cd treatments which was lost by the end of the experiment. The mussels in this study were kept in normal seawater and were not osmotically challenged. It is therefore

not surprising that there were no persistent disturbances to the major electrolytes or osmolarity which were similar to values in previous results for *M. edulis* in full strength seawater (Sheir and Handy, 2010). The absence of differences between the Cd-exposure regimes suggest that the haemolymph is osmotically well buffered against rapid changes in circulating Cd, and/or that the tissue Cd levels were too low to cause any measurable osmotic disturbance to the Na^+ concentrations in the internal organs. Cd is well known to interfere with Ca^{2+} homeostasis in freshwater organisms (Verbost et al., 1989), but this appears to be less critical in marine animals with microgram levels of Cd. In the present study, there were no effects on tissue Ca^{2+} concentrations. Burke et al. (2003) also found no effects of a 10 day exposure to much higher milligram levels of Cd (34 µg l^{-1} as free Cd^{2+} ions) on the Ca^{2+} concentrations in the hepatopancreas of *Carcinus maenas*, although there was some loss of Ca^{2+} from the posterior gill in full strength seawater.

3.3.4. Oxidative Stress and Organ Pathology during Cd Exposure

Cadmium is known to cause lipid peroxidation of cell membranes, and oxidative stress has been implicated in the toxicity of Cd to aquatic organisms, albeit often at high mg l^{-1} concentrations (Liu et al., 2009). In the present study, apart from some transient changes in TBARS (Figure 9) and total glutathione (Figure 10) concentrations in the tissues at day 6, there were no effects on either parameter by the end of the experiment. Previous work in our laboratory also observed no changes in superoxide dismutase activity in *M. edulis* during 50 µg Cd l^{-1} exposure suggesting that the concentration of Cd and short exposure duration used in the present study was not enough to cause an overt oxidative stress response. For example, Kaloyianni et al. (2009) observed increased malondialdehyde (MDA) concentration in the supernatant fraction of the

haemolymph of *M. galloprovincialis* exposed to 100 µg Cd l^{-1} for 6 days, and diminished at day 12. Géret et al. (2002) also observed an increase in MDA concentration in the digestive gland and gill after a 21 days exposure to 200 µl l^{-1} Cd. Alternatively, the decreases in relation to time may reflect short half-life in tissue, especially for TBARS (Kaloyianni et al., 2009). However, there was some evidence of a haemocyte infiltrate into the digestive gland (Figure 11), and this is likely to be part of a protective response to inflammation during Cd exposure, which can be exacerbated by inflammatory products like lipopolysaccharide, LPS (Sheir and Handy, 2010). In the present study, the quantitative histological scores indicated that the intermittent exposure had about half the haemocyte infiltrate found in the continuous exposure, suggesting the response is simply a function of the Cd exposure dose (concentration x time) rather than the exposure profile *per se*. There was also some organ pathology in the gills, mainly mild injuries with some hyperplasia in the gill filaments. This has been observed before during sub-lethal Cd exposures (Sheir and Handy, 2010), and is likely a reactive hyperplasia associated with an attempt to replace the damaged cells on the tips of the lamellae. However, there was no clear difference in the extent of these gill injuries in the Cd treatments.

3.3.5. Conclusions and Implications for Environmental Risk Assessment

Overall, the present study has shown differences in the Cd accumulation for *M. galloprovincialis* exposed intermittently or continuously to $CdCl_2$ in seawater. A concern for environmental risk assessment is whether or not the data collected from regulatory ecotoxicity tests, which use continuous exposure protocols, will be protective of intermittent contamination events. The present study has demonstrated that with equal peak concentrations of Cd, the intermittent exposure resulted in about half the Cd

accumulation in the internal organs, consistent with the idea of an additive effect of the exposure dose. It would therefore seem reasonable to use continuous exposure toxicity test data on an equivalent dose basis to predict the toxicity of an intermittent event for environmental risk assessment. Albeit, with the caveat of uncertainty that the present data is only on one species, and for short pulses of a couple of days each in seawater. Notably, the biological effects reported here for the intermittent exposure were generally either about the same, or less, than the continuous exposure regime. This is an important negative finding from a regulatory perspective. The absence of differences in biological effects between the regimes implies that the routine biological end points in regulatory tests will probably not underestimate the hazard of intermittent Cd exposure in seawater, and could be used to predict toxicity of intermittent events, at least for *M. galloprovincialis.*

Chapter 4 ***:***

Comparison of Continuous and Intermittent Exposure to Inorganic Mercury in the Blue Mussel, Mytilus galloprovincialis: Accumulation and Sub-lethal Physiological Effects

Abstract

This study aimed to compare Hg accumulation and Hg-dependent physiological responses of mussels, *M. galloprovincialis* during continuous and intermittent exposure. Mussels were exposed using a semi-static and triplicated design to either control (no added Hg) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous (daily) or intermittent (2 day exposure, 2 days in clean seawater alternately) exposure for 14 days. Tissues and haemolymph were collected and analysed for Hg accumulation and sub-lethal responses using a suite of assays. A time-dependent increase in Hg accumulation was observed in the continuous exposure, while the intermittent treatment showed step-wise changes in some tissues (e.g., gill). At the end of the experiment, the tissue Hg concentration was statistically significantly increased in the continuous compared to the intermittent for digestive gland (4 fold), gonad and remaining soft tissue (> 2 fold) but not in the gill and adductor mussel. There was no observed tissue oxidative damage at the end of the experiment as measured by the thiobarbituric acid reactive substances (TBARS) concentrations in all treatments. However, total glutathione was decreased in the gills and digestive gland of both continuous and intermittent exposure by the end of the experiment. The neutral red retention ability of the haemocytes was not affected, but total haemocyte counts were significantly decreased (< 2 fold) in the intermittent compared to the continuous exposure. Histopathological examinations showed less pathology in the gill, but high inflammation in the digestive gland of the intermittent compared to the continuous exposure. Overall, the results showed that Hg accumulation from the intermittent exposure was less than that of the continuous exposure, but the sub-lethal responses are more severe than expected in the former.

4.0. Introduction

Implementation of better contamination control measures has reduced the direct discharges of environmental contaminants into receiving waters (McCahon and Pascoe, 1990). Intermittent exposure is a more environmentally relevant means of environmental contamination (McCahon and Pascoe, 1990; Handy, 1994). The short duration of intermittent events, along with the difficulties of obtaining details of the exposure, often prevents accurate determination of toxicity threshold for adverse effects (Handy, 1994). Water quality criteria for intermittent exposure are set from the No Observable Effect Concentrations (NOECs) derived from the continuous exposure data (e.g., with pesticides), with the assumption that the response of organisms is equal to that in continuous exposure at an equivalent dose (Boxall et al., 2002). There are concerns that the NOECs derived from such continuous exposure data may under- or over-estimate toxicity for intermittent contamination events (Hickie et al., 1995; Ashauer et al., 2007a). Although some studies on intermittent exposure have focused on metals with fishes (Pascoe and Shazili, 1986; Handy, 1992) or invertebrates (Coleman, 1980; Shuhaimi-Othman and Pascoe, 2007), the reports are mainly on metal accumulation with limited information on the biological effects. A recent study from our laboratory showed that Cd accumulation by mussels, *Mytilus edulis*, was generally greater during continuous exposure than in an intermittent profile; but despite less Cd accumulation in the latter, the intermittent event was just as toxic in terms of sub-lethal biological responses (Amachree et al., 2013). Overall, there remains no consensus view on the correlation between tissue metal accumulation and response during intermittent exposure.

The ecotoxicology of the environmental fate of mercury has been extensively studied (Wolfe et al., 1998; Boening, 2000; Evers et al., 2011; Riva-Murray et al., 2011). Mercury in the earth's crust can be emitted and released into the environment

through natural processes such as weathering (Boening, 2000; Wang et al., 2004). Apart from the natural sources of Hg contamination, Wang et al. (2004) indicated six anthropogenic categories by which Hg can contaminate aquatic systems. Total Hg concentration in water bodies is highly variable ranging from values less or around 1 ng Hg l^{-1} (Soerensen et al., 2013) in open ocean or < 5 ng Hg l^{-1} in unpolluted surface water (Cossa and Fileman, 1991) to levels as high as 50 µg Hg l^{-1} in water bodies near industries discharging Hg (Rocha et al., 2013) or > 100 µg Hg l^{-1} in chloro-alkali plant waste water (Von Canstein et al., 1999). Inevitably, both the natural and anthropogenic releases of Hg into the environment are likely to be intermittent. Due to the affinity of the mercury species to particulates and organic matters, coastal sediments serve as the ultimate sink to mercury input (Mason and Lawrence, 1999) thereby putting aquatic organisms such as the filter feeding bivalves at risk (Sauvé et al., 2002).

In mammals, sub-lethal effects of continuous exposure to Hg include damage to the central nervous systems, and decreased cholinesterase activity (Wolfe et al., 1998). In marine invertebrates, the target organs for inorganic Hg exposures include the gills and digestive gland, with the gills showing the highest Hg concentration (Roesijadi et al., 1984; Canesi et al., 1999; Géret et al., 2002; Sheir et al., 2010). For example, previous studies in our laboratory on *M. edulis* with continuous laboratory exposure to 50 µg Hg l^{-1} (as $HgCl_2$) for 8 days showed gill tissue concentration of 367 µg Hg g^{-1} dry weight tissue; a 60 fold increase compared to the control values of 6 µg Hg g^{-1} dry weight tissue (Sheir et al., 2010). Sub-lethal effects of mercury in *Mytilus* species with continuous exposure profiles have been reported. Examples include an increased malondialdehyde concentration in the gill and digestive gland after 21 days exposure to 20 µ Hg g l^{-1} (Géret et al., 2002); increased DNA damage in the haemocytes after 3 days exposure to 20 µg Hg l^{-1} as $HgCl_2$ (Tran et al., 2007); increased phagocytotic ability of the haemocyte, inflammation and organ pathology after 11 days exposure to 50 µg Hg l^{-}

[1] as $HgCl_2$ (Sheir et al., 2010); reduced growth rate (Strömgren, 1982); lower filtration rate (Micallef and Tyler, 1990, Abel, 1976); generation of reactive oxygen species (ROS), genetic abnormalities in the tissues (Yap et al., 2004); tissues pathology and increased phagocytic activities in the tissues (Sheir et al., 2010); inhibition of Ca^{2+}-ATPase activity (Pattnaik et al., 2007); and disturbances to intracellular Ca^{2+} (Variengo et al., 1994). The kinetics of mercury in mussels confirm relatively fast accumulation (Roesijadi et al., 1984, Casas et al., 2004), but the clearance rate from whole mussel can be slow with a biological half-life ($t_{1/2}$) ranging from 53-293 days depending on the history and magnitude of the exposure (Riisgård et al., 1985).

During continuous exposure to contaminants, internal organs of organisms can reach equilibrium with respect to the external metal concentration (Seager and Maltby, 1989). For intermittent exposure, the equilibrium state remains unclear since in this case, the accumulation is a function of both the exposure concentration and the duration of time spent on clean water between each intermittent pulse (Handy, 1994). There is also the concern that for accumulating metals like mercury, organisms may not be able to clear body concentration between pulses. This was demonstrated in previous work (Handy, 1995) where the gills of rainbow trout and goldfish as well as the whole body concentration of total Hg in fathead minnows during a 120 h exposure showed a step-wise increase which temporally matched the pulses of exposure. This suggests that metal accumulation during intermittent exposure may be dynamic and dependent on the exposure profile.

Unlike the continuous exposure studies, sub-lethal effects of Hg during intermittent exposures are limited in shellfish and have not been documented for *M. galloprovincialis*. The present study was designed to compare Hg accumulation and measure a range of sub-lethal end points relating to the main physiological processes affected by Hg. These included osmoregulation (tissue electrolytes, plasma electrolytes

and osmotic pressure), oxidative stress parameters (lysosomal membrane damage via neutral red retention, total glutathione and thiobarbituric acid reactive substances, TBARS), and general animal health (haematology and organ pathology) during intermittent or continuous exposure to aqueous Hg.

4.1. Methodology

4.1.1. Test Organism and Acclimation

Mussels (shell length; 40-60 mm) were collected in August, 2011 from Port Quin and acclimated. The stock filtered seawater was tested daily for pH, salinity, dissolved oxygen and total ammonia as described (Sections 2.1 and 2.2).

4.1.2. Experimental Design

The experimental design is the same as that reported in chapter 3 of this thesis. Briefly, one hundred and ninety-eight (198) mussels (whole weight: 17.9 ± 0.3 g; shell length: 51.4 ± 0.4 mm; mean ± SEM) were randomly selected from the stock mussels and allocated into prior acid washed nine glass tanks containing 20 l of filtered seawater as described (section 2.2). Twenty-four hours after transfer day 0 (18 stock mussels) were collected and 12 used for Hg accumulation, histology and biochemical analysis. The remaining mussels (20 mussels/tank; 3 tanks/treatment; total of 60 mussels/treatment) were exposed using a semi-static exposure regime with 100% water change every 24 h, to either a control (filtered seawater only, no added Hg) or 50 µg Hg l^{-1} as $HgCl_2$ for both continuous and intermittent regimes. The concentration of 50 µg Hg g^{-1} as $HgCl_2$ was selected as a known concentration that is sub-lethal to mussels based on previous experiments in our (Sheir et al., 2010). Dosing was achieved by

adding 1 ml of 1g Hg l^{-1} as $HgCl_2$ stock solution to treatment tanks (containing 20 l of filtered seawater) to give a nominal concentration of 50 µg Hg l^{-1}.

Filtered seawater quality was analysed daily and there were no observed significant differences between glass aquaria for all treatments (ANOVA, $p > 0.05$). Values were (means ± SEM, n = 42 samples/treatment); pH (7.8 ± 0.2); salinity (35.5 ± 0.1 ppt); dissolved oxygen, DO (9.7 ± 1.0 mg l^{-1}) and total ammonia (1.0 ± 0.1 mg l^{-1}). Water samples for Hg concentration were also collected daily immediately after and before test media renewal. Background Hg concentration in the control filtered seawater (means, n = 42, Figure 14) was below the detection limit of the instrument (0.28 ± 0.05 µg l^{-1}). Sampling at 2, 4, 6, 8, 10, 12, and 14 days were performed exactly as that described (Section 3.1.2).

4.1.3. Heamolymph Extraction and Tissue Collection

Haemolymph extraction and tissue collection were performed exactly as described (Section 2.4).

4.1.4. Trace Metal Analysis

Tissues Hg concentration and trace elements composition were analysed according Sheir et al. (2010) as described (Section 2.5). All seawater samples were analysed for Hg by ICP-MS with a detection limit of 0.28 ± 0.05 µg Hg l^{-1}. The procedural detection limit for Hg analysis in tissue digests on the ICP-OES was and 18.46 µg Hg l^{-1} and was derived from three times the standard deviation of the procedural blank. For a typical 0.1g of tissue the detection limit equates to 0.92 µg Hg g^{-1} dry weight tissues.

4.1.5. TBARS and Total Glutathione Analysis

TBARS and total glutathione were performed exactly as described (Sections 2.7.1 and 2.7.2 respectively). Preliminary measurements of total glutathione in the gills of Hg-exposed mussels gave apparently very low levels (below the detection limit) and to aid data interpretation, time course test was performed on gill tissue from clean mussels that were excised and spiked with Hg. Briefly, gill tissue was collected from three clean mussels and pooled (Figure 12). Tissues were weighed, divided into 2 halves (approximately 0.3g each) and homogenised in 5 volumes of iced-cold buffer (as above). One of the halves was used as a control (no added Hg) and the other spiked with Hg. Spiking was achieved by adding 0.3 ml of 1 g Hg l^{-1} as $HgCl_2$ stock solution to give a tissue concentration of 1000 µg Hg g^{-1} tissue. Tissues were then centrifuged and supernatants assayed as described above. GSH concentrations in the supernatants were determined by reference to the rate of change of absorbance of the standard, after substracting the rate of change of absorbance for the blank. The kinetic assay was not distinguishable from that of the rate of change in absorbance of a 1 µmol l^{-1} GSH standard recorded for up to 5 min at 412 nm, and the calculated detection limit was 1.2 µmol l^{-1}. For a typical 0.1 g of tissue the detection limit equates to 0.12 nmol GSH g^{-1} ww tissue. Total glutathione in the spiked gill tissue were not detected, but the unspiked control showed total glutathione concentration of 2.60 nmol g^{-1} ww (Figure 13).

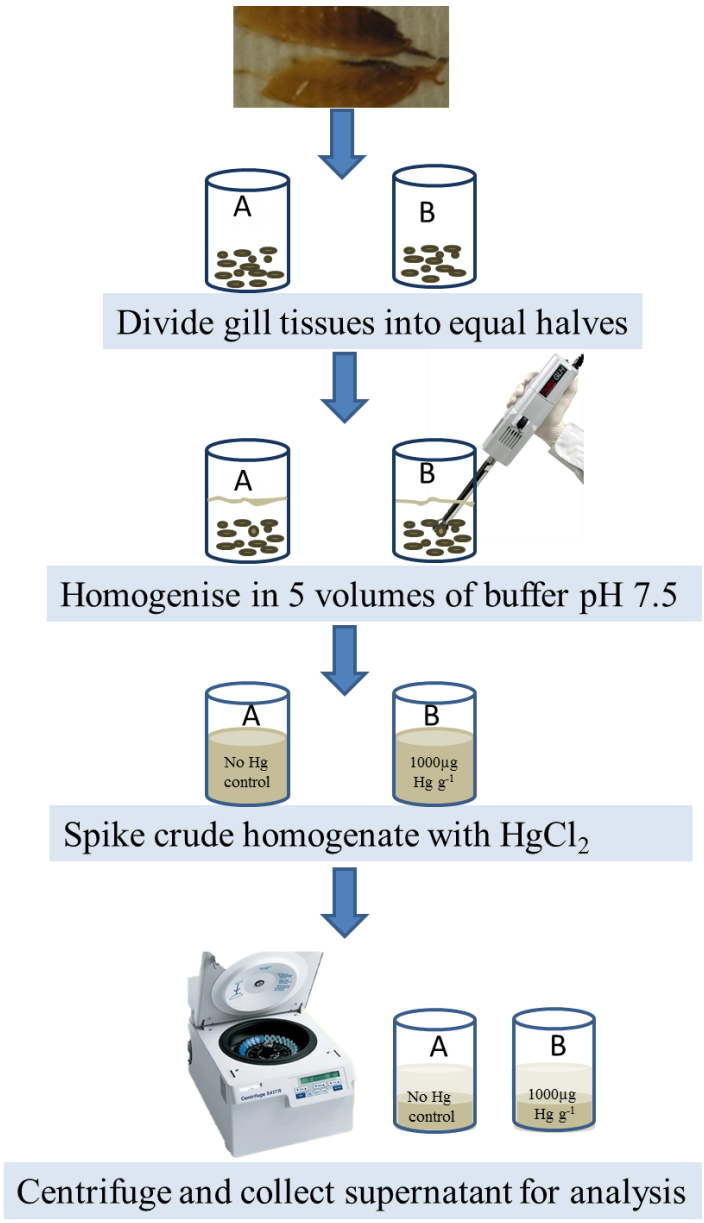


Figure 12: Investigating the apparent low levels of total glutathione in gill tissue exposed to 50 µg Hg l^{-1} as $HgCl_2$ for up to 14 day in continuous and intermittent exposure. Gill tissues collected from clean mussels were divided into 2 equal halves and homogenised. One half was not spiked (no added Hg) but the other spiked with 0.3 ml of 1 g l^{-1} Hg as $HgCl_2$ stock solution to give 1000 µg Hg g^{-1} tissue. Thereafter, homogenates were centrifuged and supernatant analysed for tissue total glutathione concentration.

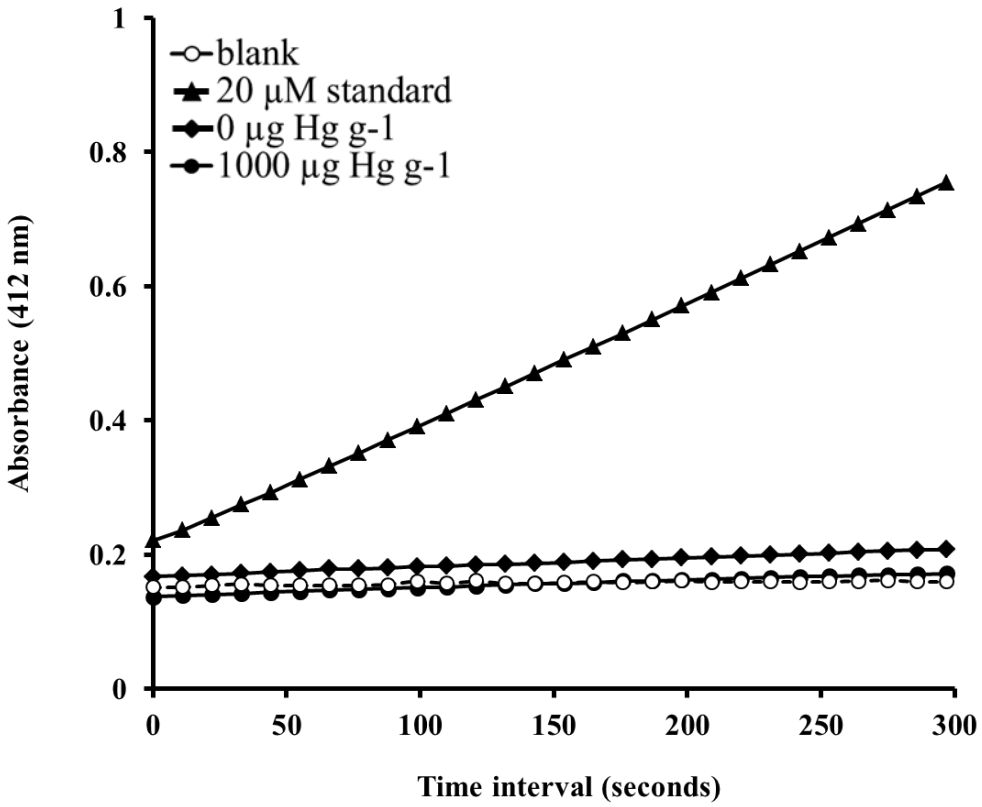


Figure 13: Representative time courses of the change in absorbance over time in the total glutathione assay. The close circle on solid line represents the blank, Mili-Q water; the closed triangle on solid line represents the 20 µmol l^{-1} GSH standard; the diamond on solid line represents the control, 0 µg Hg g^{-1} tissue (no added Hg) and the open circle on broken line represents a spike of 0.3 ml of 1 g l^{-1} Hg as $HgCl_2$ stock solution equivalent to 1000 µg Hg g^{-1} tissue. Data are means, n = 3 wells per treatment. The calculated detection limit for a 1 µmol l^{-1} GSH standard was 1.2 µmol l^{-1}. For a typical 0.1 g of tissue the detection limit equates to 0.12 nmol GSH g^{-1} ww tissue.

4.1.6. Neutral Red Retention and Total Haemocyte Count

The effect of Hg on the lysosomal integrity of the haemocytes was assessed by the neutral red retention (NRR). Haemocyte count was also performed as described (Section 2.9 and 2.10).

4.1.7. Plasma Ion, Osmotic Pressure and Glucose Assay

Na^+, K^+, osmotic pressure and glucose in the plasma were measured exactly as described (Section 2.11 and 2.12).

4.1.8. Histological Examinations

Histological investigations were conducted exactly as described as (Section 2.6).

4.1.9. Calculations and Statistical Analysis

Statistical analyses were performed on all data by StatGraphics Plus for windows version 5.1 as described (Section 2.13). The IBM SPSS Statistics version 20 was used to analyse and draw graphs for correlation relationship between Hg accumulation and total glutathione concentration of the tissues.

4.2. Results

4.2.1. Aqueous Exposure to Mercury and Tissue Accumulation

The exposure profiles were confirmed by the measured Hg concentrations in the glass aquaria (Figure 14). Background Hg concentration in the control filtered seawater (means, n = 42, Figure 14) was below the detection limit of the instrument (0.28 ± 0.05 µg Hg l^{-1}, n = 6). The nominal 50 µg Hg l^{-1} exposure concentration was confirmed by the measured total concentration in the glass aquaria and were (means ± SEM) 47.4 ± 0.6 µg Hg l^{-1} (n = 42) and 48.1 ± 1.1 µg Hg l^{-1} (n = 21) for continuous and intermittent exposure respectively. However, the concentration of the Hg in the tanks immediately before the daily water change were decreased with mean values (means ± SEM, µg Hg l^{-1}) 5.8 ± 1.7 and 7.1 ± 1.4 for the continuous and intermittent exposure, respectively. This represents 87 and 85% decrease in the total Hg concentration over 24 h during an exposure phase for the continuous and intermittent exposure, respectively. This can be explained by the numerous binding sites for Hg^{2+} within the experimental system, for example the surface of the experimental tanks. These values are similar to that seen in Tran et al. (2007).

Aqueous exposure of *M. galloprovincialis* to 50 µg Hg l^{-1} as $HgCl_2$ did not result in mortality during the experiment for all treatments including controls, confirming the sub-lethal exposure concentration.

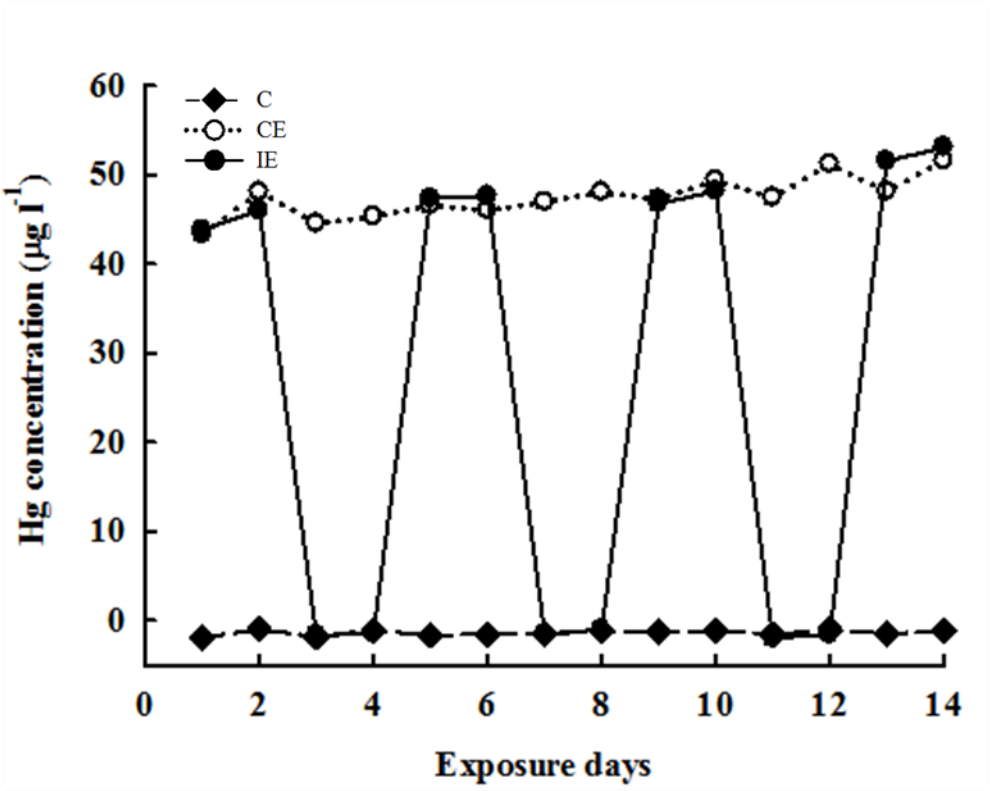


Figure 14: The mercury concentration profile in seawater after 14 days exposure to control, C (no added Hg, diamonds on dashed line) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous, CE (open circles on dotted line) or intermittent, IE (closed circle on solid line) exposure. Samples were collected at the beginning of each exposure day, immediately after the renewal of the test media. Measurements of Hg just before renewal of the test media are not included for clarity. Data are means, µg l^{-1} for n = 3 tests per treatment at each exposure day. The detection limit (0.28 µg l^{-1}) was used to replace all values below detection limit. Error bars are not shown for clarity. The line on the intermittent exposure was drawn to emphasise the intermittent phases, it does not imply a gradual change in exposure concentration between dosing within each pulse, as full water changes were done manually in each tank.

4.2.2. Tissue Hg Accumulation

Tissue-specific differences were seen in Hg accumulation with the highest concentrations in the gills > digestive gland > gonads > remaining soft tissue > adductor muscle (Figure 15). Exposure to Hg using either continuous or intermittent regimes resulted in increased tissue accumulation compared to the controls (ANOVA, $p < 0.001$). For example, at the end of the experiment Hg concentration in the gill (means ± SEM, $n = 6$, µg Hg g^{-1} dry weight) was 1.2 ± 0.7, 1732.2 ± 132.5 and 1350.8 ± 175.8 for control, continuous and intermittent exposure respectively. Two-way ANOVA showed statistically significant treatment and time interaction (2-way ANOVA, $p \leq 0.001$) for all tissues examined.

In the continuous exposure, a time-dependent curvilinear pattern of accumulation was seen in the gonad and remaining soft tissue up to the end of the experiment (Figure 15). The adductor muscle showed a non-statistically significant increasing trend over time phase accumulation pattern. There was a transient plateau from day 4 to 10 in the gill and digestive gland.

Like the continuous exposure, tissues in the intermittent regime also showed an increasing trend of Hg accumulation in all tissues examined apart from the gill. The gill showed statistically significant step-wise increases demonstrated by a clear alternating uptake and clearance trend corresponding with the exposure profile (2 days exposure: 2 days in clean seawater, Figure 15). Apart from the initial uptake phase (up to day 4), the adductor muscle and gonad, showed a plateau up to the end of the experiment. There was no statistically significant difference in Hg concentration in the digestive gland and remaining soft tissue from day 10 up to the end of the experiment (Figure 15).

Hg tissue concentration in the continuous was significantly increased; 4 fold for digestive gland and 2 fold for gonad and remaining soft tissue compared to the intermittent (ANOVA, $p < 0.001$). There were no significant differences between the

continuous and the intermittent exposure for gill and adductor mussel at the end of the experiment (ANOVA, $p > 0.001$).

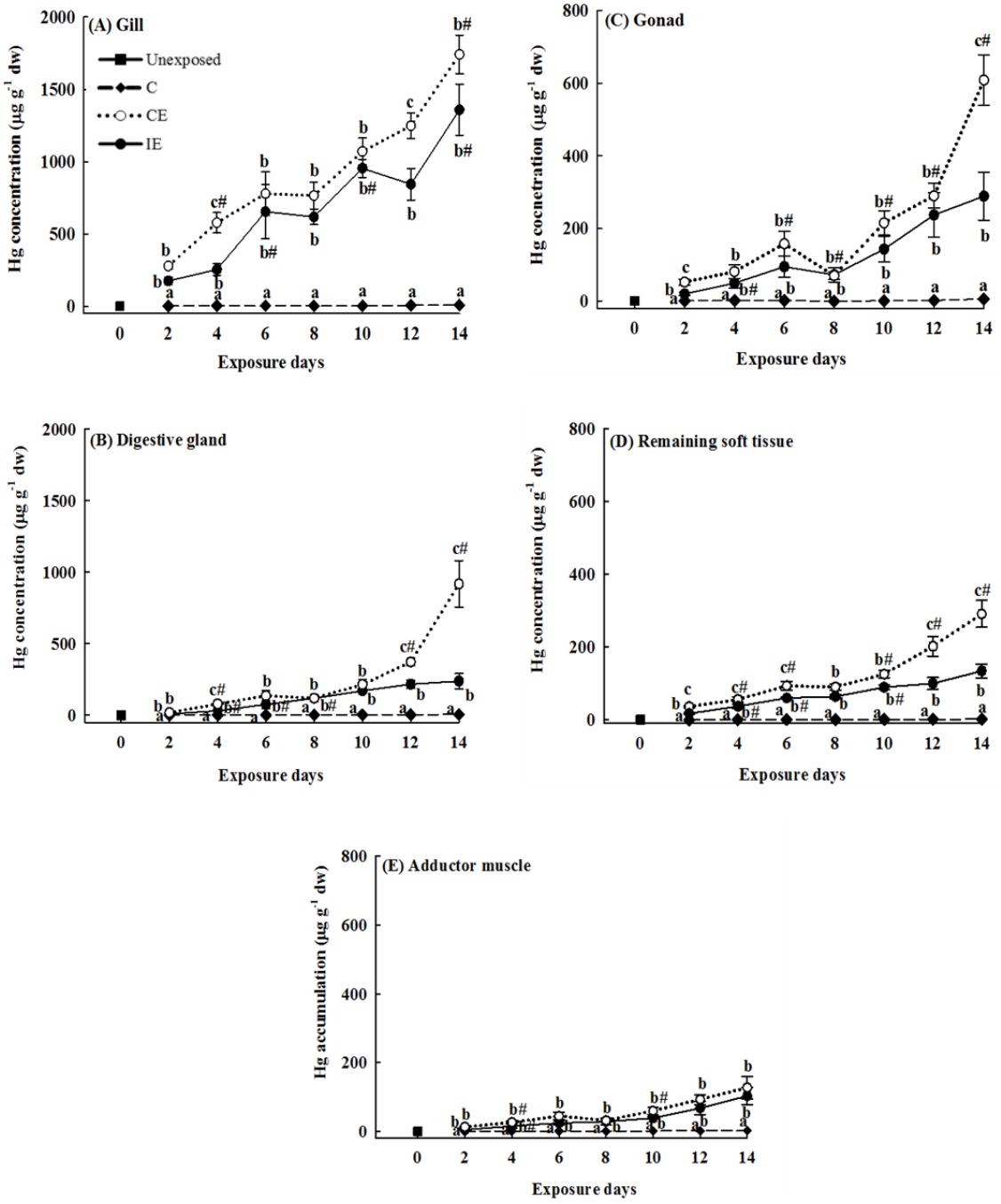


Figure 15: Mercury concentrations in the (A) gill (B) digestive gland, (C) remaining soft tissue, (D) gonad and (E) posterior adductor muscle of *M. galloprovincialis* after 14 days exposure to control, C (no added Hg, diamonds on dashed line) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous, CE (open circles, dotted lines) or intermittent, IE (closed circles, solid lines) exposure. The black square at time zero represents the background Cd concentration in unexposed (initial) mussels at the start of the experiment. Data are means ± SEM, µg Hg g^{-1} dry weight tissue, n = 6 mussels per treatment at each exposure day. Different letters within the exposure day indicates a significant treatment effect (ANOVA, $p < 0.05$). # indicates a significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, initial mussel stock) (ANOVA, $p < 0.05$).

4.2.3. Effects of Hg Exposure on Haemolymph Chemistry and Ionic Regulation

Total haemocytes counts, neutral red retention and plasma glucose assays were performed at the start, middle and the end of the experiment (Table 12). Hg exposure to either continuous or intermittent exposure resulted in treatment-dependent statistically significant differences in the total haemocyte counts compared to the control (ANOVA, $p < 0.05$) by the end of the experiment. There was an increase in the total haemocytes count in continuous exposure and a decrease in the intermittent exposure compared to the control (Table 12). There was no statistically significant treatment effect on the neutral red retention ability of the haemocytes in either continuous or intermittent exposure compared to the control, although there were some statistically significant time-dependent differences within treatments (ANOVA, $p < 0.05$, Table 12). By the end of the experiment, the values of the neutral red retention ability of the haemocytes in the intermittent exposure elevated to > 2 fold to that of the previous time point (day 6, Table 12).

Glucose concentration in the cell-free haemolymph (plasma) was generally low throughout the experiment (< 1 mmol l^{-1}). There was no significant treatment- or time-dependent effect (ANOVA, $p > 0.05$) on the glucose concentration in all treatments including controls. Likewise, the osmotic pressure was not significantly affected by time or Hg treatments (ANOVA, $p > 0.05$, Table 12). Plasma Na^+ and K^+ exhibited statistically significant transient changes at some time points (ANOVA, $p < 0.05$). However, by the end of the experiment values remained within the normal range for marine mussels (means, $n = 6$, mmol l^{-1}), 448.7-583.5 and 10.5-12.2 for Na^+ and K^+ respectively (ANOVA, $p <0.05$, Table 12).

Table 12: Total haemocyte counts (THC), neutral red retention (NRR) and solute concentration in the cell-free haemolymph *from M.galloprovincialis* after 14 days exposure to 0 (no added Hg control) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous or intermittent exposures.

Parameters	Treatments	Exposure days							
		0	2	4	6	8	10	12	14
THC	Control	0.76 ± 0.3			2.57 ± 0.8+				1.90 ± 1.0a+
(*10^6 cells ml^{-1})	Continuous	-			2.57 ± 0.5+				2.12 ± 0.2b#+
	Intermittent	-			2.56 ± 0.7+				1.53 ± 0.7c#+
NRR	Control	5.69 ± 1.6			1.87 ± 0.5#+				2.75 ± 0.6+
(OD 106 $cells^{-1}$)	Continuous	-			2.73 ± 0.6+				3.79 ± 0.7+
	Intermittent	-			1.80 ± 0.3+				5.20 ± 1.6#+
Glucose	Control	0.53 ± 0.2			0.51 ± 0.1				0.51 ± 0.1
(mmol l^{-1})	Continuous	-			0.51 ± 0.1				0.50 ± 0.1
	Intermittent	-			0.50 ± 0.1+				0.52 ± 0.1

Continuation of Table 12: Total haemocyte counts (THC), neutral red retention (NRR) and solute concentration in the cell-free haemolymph *from M.galloprovincilais*

Parameters	Treatments	Exposure days							
		0	2	4	6	8	10	12	14
Osmotic pressure (mosmol Kg^{-1})	Control	1045.3 ± 10.8	1100.7 ± 30.2	1060.5 ± 24.3	1051.7 ± 15.4	1060.8 ± 12.8a	1108.5 ± 16.1	1101.2 ± 17.9	1073.3 ± 12.1
	Continuous	-	1043.2 ± 12.3	1059.5 ± 26.9	1082.5 ± 35.6	1167.8 ± 33.1b#+	1131.7 ± 35.6+	1063.3 ± 23.7	1099.8 ± 22.6
	Intermittent	-	1078.5 ± 18.5	1102.7 ± 46.0	1060.3 ± 13	1084.2 ± 34.1ab	1090.8 ± 17.2	1101.2 ± 23.7	1038.3 ± 20.9
Na^+ (mmol l^{-1})	Control	484.2 ± 7.6	510.1 ± 10.3	448.7 ± 5.5#+	523.3 ± 4.6+	533.7 ± 13.7a+	521.9 ± 18.8+	507.7 ± 8.0	495.5 ± 15.5#
	Continuous	-	477.5 ± 7.8	471.1 ± 8.6	524.8 ± 16.5+	583.5 ± 16.9b#+	539.1 ± 21.9#+	499.9 ± 16.4	495.1 ± 13.8#
	Intermittent	-	476.3 ± 12.6	451.7 ± 25.8	513.6 ± 9.2+	508.9 ± 11.7a	502.7 ± 7.3	500 ± 9.9	490.7 ± 20.1#
K^+ (mmol l^{-1})	Control	11.3 ± 0.2	12.2 ± 0.2a	9.9 ± 0.2#+	12.0 ± 0.4	11.1 ± 0.4	11.5 ± 0.6	11.7 ± 0.2	11.8 ± 0.3#
	Continuous	-	10.9 ± 0.2b	10.7 ± 0.3	12.0 ± 0.6	11.9 ± 0.4	11.8 ± 0.3	12.0 ± 0.3	12.0 ± 0.8
	Intermittent	-	10.92 ± 0.3b	10.6 ± 0.7	11.6 ± 0.2	11.2 ± 0.3	10.7 ± 0.2	10.94 ± 0.2	10.5 ± 0.4

Data are means ± SEM, $n = 6$ mussels per treatment per exposure day. Different letters within each exposure day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + indicates significant time effect compared to day 0 (stock mussels, ANOVA, $p < 0.05$). Note, only the total haemocyte data were non-parametric thus Kruskal-Wallis was used. Note THC represents total haemocyte counts; NRR represents neutral red retention; OD represents optical density.

The gill, digestive gland, gonad, remaining soft tissue and adductor muscle were also analysed for major electrolytes (Na^+, K^+, Ca^{2+}, and Mg^{2+}). There was no overall treatment-dependent effect on tissue Na^+ concentrations (ANOVA, $p < 0.05$, Table 13). However, the gill showed statistically significant transient decrease in the continuous compared to the control or intermittent exposure in Na^+ concentration on day 8 which was lost at the end of the experiment (Table 13). Only the remaining soft tissue (RST) showed treatment-dependent elevations compared to the control at the end of the experiment (Table 13). Hg exposure to either continuous or intermittent mode showed some transient but significant changes at some time points in K^+ concentration in all tissues examined (Table 14). At the end of the experiment all the tissues showed significant elevations in K^+ concentration in the treated groups compared to the control (ANOVA, $p < 0.05$), but there were no difference between the continuous and the intermittent exposure (Table 14). There were no overall treatment effects in all tissues for Ca^{2+} or Mg^{2+} concentrations (ANOVA or kruskal-Wallis, $p > 0.05$, Tables 15 and 16 respectively). There were however, some time-dependent changes at various time points (data not shown). At the end of the experiment, only the remaining soft tissue and adductor muscle showed treatment-dependent significant elevation in Ca^{2+} (Kruskal-Wallis, $p < 0.05$, Table 15) and Mg^{2+} (ANOVA, $p < 0.05$, Table 16) concentration compared to the control.

Table 13: Na^+ concentrations (µmol g^{-1} dry weight tissue) in *M.galloprovincialis* after 14 days exposure to 0 (no added Hg control) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous or intermittent exposures.

Tissue	Treatments	Exposure days							
		0	2	4	6	8	10	12	14
Gill	Control	1302.0 ± 47.1	1382.0 ± 39.1	1601.6 ± 47.9#+	1501.7 ± 59.8+	1340.7 ± 115.1a	1344.4 ± 111.4	1279.5 ± 43.9a	1598.9 ± 137.4#+
	Continuous	-	1451.6 ± 53.3+	1456.5 ± 22.2+	1349.2 ± 61.9	1188.7 ± 33.7b#	1132.1 ± 34.7+	1142.6 ± 39.4b+	1682.1 ± 68.4#+
	Intermittent	-	1495.4 ± 60.8	1551.2 ± 79.4+	1427.7 ± 233.4	1305.0 ± 17.7a	1149.2 ± 76.2#	1037.6 ± 44.9b#+	1599.8 ± 67.5#+
Digestive gland	Control	590.4 ± 26.7	676.6 ± 60.5	842.5 ± 45.2+	563.8 ± 45.1#	686.2 ± 55.8+	695.6 ± 37.6	693.7 ± 44.3+	784.0 ± 79.8+
	Continuous	-	668.9 ± 45.8	712.8 ± 106.7	732.6 ± 47.1	667.0 ± 75.6	553.2 ± 45.2	731.6 ± 44.3#	975.9 ± 89.9#+
	Intermittent	-	618.2 ± 45.4	740.0 ± 35.7#+	749.4 ± 119.7#	597.2 ± 17.3	664.0 ± 61.3	663.6 ± 38.1+	914.0 ± 74.5#+
Gonad	Control	1055.9 ± 148.7	955.7 ± 213.7	1285.6 ± 204.7	823.5 ± 167.4	1032.5 ± 195.0	822.0 ± 146.1	930.8 ± 143.8	1222.3 ± 120.3
	Continuous	-	1005.5 ± 180.6	879.4 ± 236.2	1278.8 ±168.7#	690.4 ± 119.4	764.5 ± 113.6	1255.6 ± 93.5	1444.1 ± 189.0
	Intermittent	-	687.0 ± 66.5	1277.1 ± 234.9	1116.1 ± 193.0	715.5 ± 94.6	881.3 ± 249.8	960.8 ± 181.1	1073.4 ± 150.5
Remaining soft tissue	Control	901.5 ± 62.0	904.4 ± 99.7	1168.6 ± 53.3	910.8 ± 41.2	1079.5 ± 107.6a	925.8 ± 75.6	927.2 ± 69.0	831.5 ± 163.9a
	Continuous	-	1058.5 ± 69.6	1033.5 ± 132.3	1030.7 ± 179.0	804.0 ± 33.7b	829.9 ± 30.9	1045.6 ± 31.7+	1413.2 ±134.6b#+
	Intermittent	-	834.5 ± 69.2	1270.6 ± 234.9#+	1051.5 ± 24.8	876.9 ± 35.6b#	910.3 ± 83.4	973.2 ± 98.1	1264.5 ±134.1b#+
Adductor muscle	Control	544.7 ± 50.7	524.5 ± 16.2a	676.6 ± 60.8	565.2 ± 65.6	545.2 ± 60.6	496.6 ± 39.2	549.7 ± 36.5	580.7 ± 74.3
	Continuous	-	612.9 ± 20.3b	582.4 ± 59.5	739.8 ± 78.1+	513.2 ± 513.2#	497.2 ± 13.9	557.6 ± 85.7	943.2 ± 104.8#+
	Intermittent	-	431.7 ± 70.0a	578.6 ± 35.7	553.4 ± 65.6	533.3 ± 42.3	477.1 ± 45.5	515.1 ± 32.6	895.5 ± 181.69#+

Data are means ± SEM, $n = 6$/ treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to day 0 (stock mussels, ANOVA, $p < 0.05$).

Table 14: K^+ concentrations (µmol g^{-1} dry weight tissue) in *M. galloprovincialis* after 14 days exposure to 0 (no added Hg control) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous or intermittent exposures.

Tissue	Treatments	Exposure days							
		0	2	4	6	8	10	12	14
Gill	Control	1302.0 ± 47.1	1382.0 ± 39.1	1601.6 ± 47.9#+	1501.7 ± 59.8+	1340.7 ± 115.1a	1344.4 ± 111.4	1279.5 ± 43.9a	1598.9 ± 137.4#+
	Continuous	-	1451.6 ± 53.3+	1456.5 ± 22.2+	1349.2 ± 61.9	1188.7 ± 33.7b#	1132.1 ± 34.7+	1142.6 ± 39.4b+	1682.1 ± 68.4#+
	Intermittent	-	1495.4 ± 60.8	1551.2 ± 79.4+	1427.7 ± 233.4	1305.0 ± 17.7a	1149.2 ± 76.2#	1037.6 ± 44.9b#+	1599.8 ± 67.5#+
Digestive gland	Control	590.4 ± 26.7	676.6 ± 60.5	842.5 ± 45.2+	563.8 ± 45.1#	686.2 ± 55.8+	695.6 ± 37.6	693.7 ± 44.3+	784.0 ± 79.8+
	Continuous	-	668.9 ± 45.8	712.8 ± 106.7	732.6 ± 47.1	667.0 ± 75.6	553.2 ± 45.2	731.6 ± 44.3#	975.9 ± 89.9#+
	Intermittent	-	618.2 ± 45.4	740.0 ± 35.7#+	749.4 ± 119.7#	597.2 ± 17.3	664.0 ± 61.3	663.6 ± 38.1+	914.0 ± 74.5#+
Gonad	Control	1055.9 ± 148.7	955.7 ± 213.7	1285.6 ± 204.7	823.5 ± 167.4	1032.5 ± 195.0	822.0 ± 146.1	930.8 ± 143.8	1222.3 ± 120.3
	Continuous	-	1005.5 ± 180.6	879.4 ± 236.2	1278.8 ±168.7#	690.4 ± 119.4	764.5 ± 113.6	1255.6 ± 93.5	1444.1 ± 189.0
	Intermittent	-	687.0 ± 66.5	1277.1 ± 234.9	1116.1 ± 193.0	715.5 ± 94.6	881.3 ± 249.8	960.8 ± 181.1	1073.4 ± 150.5
Remaining soft tissue	Control	901.5 ± 62.0	904.4 ± 99.7	1168.6 ± 53.3	910.8 ± 41.2	1079.5 ± 107.6a	925.8 ± 75.6	927.2 ± 69.0	831.5 ± 163.9a
	Continuous	-	1058.5 ± 69.6	1033.5 ± 132.3	1030.7 ± 179.0	804.0 ± 33.7b	829.9 ± 30.9	1045.6 ± 31.7+	1413.2 ±134.6b#+
	Intermittent	-	834.5 ± 69.2	1270.6 ± 234.9#+	1051.5 ± 24.8	876.9 ± 35.6b#	910.3 ± 83.4	973.2 ± 98.1	1264.5 ±134.1b#+
Adductor muscle	Control	544.7 ± 50.7	524.5 ± 16.2a	676.6 ± 60.8	565.2 ± 65.6	545.2 ± 60.6	496.6 ± 39.2	549.7 ± 36.5	580.7 ± 74.3
	Continuous	-	612.9 ± 20.3b	582.4 ± 59.5	739.8 ± 78.1+	513.2 ± 513.2#	497.2 ± 13.9	557.6 ± 85.7	943.2 ± 104.8#+
	Intermittent	-	431.7 ± 70.0a	578.6 ± 35.7	553.4 ± 65.6	533.3 ± 42.3	477.1 ± 45.5	515.1 ± 32.6	895.5 ± 181.69#+

Data are means ± SEM, $n = 6$/ treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to day 0 (stock mussels, ANOVA, $p < 0.05$).

Table 15: Ca^{2+} concentrations (µmol g^{-1} dry weight tissue) in *M. galloprovincialis* after 14 days exposure to 0 (no added Hg control) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous or intermittent exposures.

Tissue	Treatments	Exposure days							
		0	2	4	6	8	10	12	14
Gill	Control	46.5 ± 1.7	51.3 ± 1.3	59.9 ± 5.1	55.1 ± 2.9	47.6 ± 3.5	52.8 ± 4.3a	52.9 ± 2.9a	56.2 ± 4.0
	Continuous	-	69.2 ± 16.7+	51.7 ± 0.9+	49.7 ± 2.9	52.7 ± 7.3	44.7 ± 1.0a	45.3 ± 2.3a	69.7 ± 6.7#+
	Intermittent	-	53.6 ± 3.7	53.3 ± 2.3+	54.7 ± 9.1	51.6 ± 1.8	44.8 ± 2.6b	41.8 ± 1.8a	64.6 ± 7.4#
Digestive gland	Control	28.7 ± 1.3	42.9 ± 8.8	39.7 ± 2.4	28.3 ± 2.4	30.2 ± 2.2	34.0 ± 2.7	49.2 ± 12.8	41.4 ± 4.1
	Continuous	-	35.8 ± 5.2	33.1 ± 2.7	37.3 ± 2.9	38.0 ± 5.1	34.4 ± 4.2	46.7 ± 9.6	46.2 ± 2.5
	Intermittent	-	29.4 ± 2.3	39.1 ± 1.0#+	40.7 ± 6.0	37.3 ± 3.0	39.1 ± 4.1	33.8 ± 2.3	49.4 ± 8.4+
Gonad	Control	42.0 ± 4.3	62.9 ± 14.6	51.3 ± 5.5	34.4 ± 5.4	46.1 ± 9.3	44.1 ± 10.1	64.5 ± 14.0	69.4 ± 15.1
	Continuous	-	64.5 ± 20.1	47.7 ± 11.3	76.8 ± 21.1	33.1 ± 4.8	33.8 ± 5.1	72.3 ± 10.5	62.2 ± 7.5
	Intermittent	-	43.5 ± 13.3	64.4 ± 16.4	63.9 ± 15.9	39.1 ± 2.4	48.3 ± 11.1	46.5 ± 7.3	63.9 ± 18.0
Remaining soft tissue	Control	72.9 ± 5.9	65.2 ± 10.7	70.2 ± 4.4	50.4 ± 4.3	52.5 ± 6.1	74.1 ± 11.9	81.4 ± 18.4	45.5 ± 8.9a
	Continuous	-	64.4 ± 3.5	80.1 ± 11.4	55.7 ± 9.4	78.6 ± 16.3	45.1 ± 7.6	53.0 ± 9.1	107.7 ± 14.5b#+
	Intermittent	-	46.2 ± 4.3	68.4 ± 12.8	75.4 ± 9.4	105.9 ± 34.8	52.0 ± 5.8#	99.1 ± 28.8#	113.7 ± 22.0b
Adductor muscle	Control	30.2 ± 3.7	34.8 ± 5.1a	53.1 ± 15.8	37.2 ± 7.3	29.0 ± 5.2	34.0 ± 8.2	37.4 ± 3.8	36.8 ± 4.8a
	Continuous	-	36.4 ± 4.4a	40.1 ± 6.0	44.6 ± 5.6	29.6 ± 4.5#	28.2 ± 2.3	34.6 ± 8.2	62.7 ± 12.4b#+
	Intermittent	-	18.2 ± 2.5b	33.6 ± 3.1#	45.9 ± 10.3	67.6 ± 30.8	25.4 ± 1.3	47.0 ± 13.5#	77.0 ± 24.1b#+

Data are means ± SEM, n = 6/ treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to day 0 (stock mussels, ANOVA, $p < 0.05$).

Table 16: Mg^{2+} concentrations (µmol g^{-1} dry weight tissue) in *M. galloprovincialis* after 14 days exposure to 0 (no added Hg control) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous or intermittent exposures.

Tissue	Treatments	Exposure days							
		0	2	4	6	8	10	12	14
Gill	Control	177.1 ± 7.3	190.4 ± 26.4	216.7 ± 6.0#+	201.6 ± 6.4+	192.9 ± 16.0	194.4 ± 12.8	186.6 ± 6.3a	229.2 ± 18.0#+
	Continuous	-	195.9 ± 6+	198.4 ± 3.0+	189.9 ± 7.1	175.5 ± 4.5	173.6 ± 4.7	173.0 ± 5.2a	232.1 ± 8.3#+
	Intermittent	-	202.0 ± 8.1	206.3 ± 10.8+	209.9 ± 34.5	196.3 ± 2.8+	173.6 ± 10.1	158.4 ± 5.1b#	228.2 ± 9.2#+
Digestive gland	Control	87.1 ± 3.1	96.9 ± 7.3	119.0 ± 5.3#+	85.1 ± 6.7a#	103.8 ± 8.6#	102.1 ± 3.8	104.3 ± 5.7	119.2 ± 9.4#+
	Continuous	-	97.4 ± 5.9	101.3 ± 12.5	107.6 ± 5.6b+	101.1 ± 8.3	87.9 ± 5.8	110.7 ± 4.9#+	141.1 ± 11.3#+
	Intermittent	-	87.8 ± 6.8	105.5 ± 6.6	115.5 ± 18.3b+	96.0 ± 2.8#	103.1 ± 7.2	102.1 ± 5.0	131.3 ± 10.5#+
Gonad	Control	133.5 ± 16.7	129.6 ± 26.4	168.6 ± 22.5	111.5 ± 20.4	142.9 ± 25.3	116.3 ± 17.4	129.7 ± 17.3	177.2 ± 18.3
	Continuous	-	135.7 ± 21.6	116.4 ± 30.1	161.9 ± 17.1#	98.2 ± 14.7#	113.1 ± 16.7	177.8 ± 15.1#	224.7 ± 38.8+
	Intermittent	-	93.0 ± 9.7	163.8 ± 32.4	155.3 ± 25.8	105.9 ± 12.3	124.2 ± 29.8	137.1 ± 23.1	191.9 ± 47.3
Remaining soft tissue	Control	122.1 ± 6.6	122.3 ± 12.2	155.2 ± 6.5	122.7 ± 5.4	150.5 ± 12.1a	130.7 ± 8.8	133.6 ± 8.4	116.0 ± 22.4a
	Continuous	-	142.5 ± 8.1	138.9 ± 16.9	135.4 ± 22.1	115.2 ± 4.0b	119.9 ± 4.1	152.3 ± 4.5#+	187.4 ± 16.0b#+
	Intermittent	-	114.1 ± 8.3	136.6 ± 7.5	148.3 ± 3.2+	127.0 ± 4.4a	129.5 ± 9.1	142.8 ± 13.6	173.2 ± 17.3b#+
Adductor muscle	Control	78.8 ± 5.4	77.3 ± 1.8a	95.9 ± 7.1	81.6 ± 7.3a	82.5 ± 6.4	76.2 ± 4.1	82.7 ± 4.5	88.0 ± 9.6a
	Continuous	-	88.4 ± 2.4b	82.9 ± 6.6	101.0 ± 8.8b#+	77.3 ± 2.2	78.8 ± 1.4	85.2 ± 10.0	131.1 ± 12.3b#+
	Intermittent	-	61.9 ± 9.4a	82.2 ± 4.6	82.1 ± 9.3a	83.5 ± 5.1	76.0 ± 4.7	80.0 ± 4.1	128.1 ± 21.6b#+

Data are means ± SEM, n = 6/ treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to day 0 (stock mussels, ANOVA, $p < 0.05$).

4.2.4. Effects of Hg exposure on TBARS

Continuous exposure to Hg *in M. galloprovincialis* resulted in some transient treatment-dependent increase in TBARS concentration on day 6 in all tissues compared to the control or intermittent exposure (ANOVA, $p < 0.05$), which were lost at the end of the experiment (Figure 16). The TBARS concentration in the continuous exposure on day 6 showed percentage (%) increases of 50, 55, 70 and 43% in gill, digestive gland, gonad and adductor muscle respectively compared to the intermittent exposure. However at the end of the experiment, there were no significant differences in TBARS concentration in the gill, digestive gland, gonads and adductor muscle in all treatments including the controls (Figure 16). Pearson's correlation coefficient (r) did not show any relationship between tissue Hg and TBARS concentrations for the gill (0.11), digestive gland (0.15), gonad (-0.02), and adductor muscle (0.02).

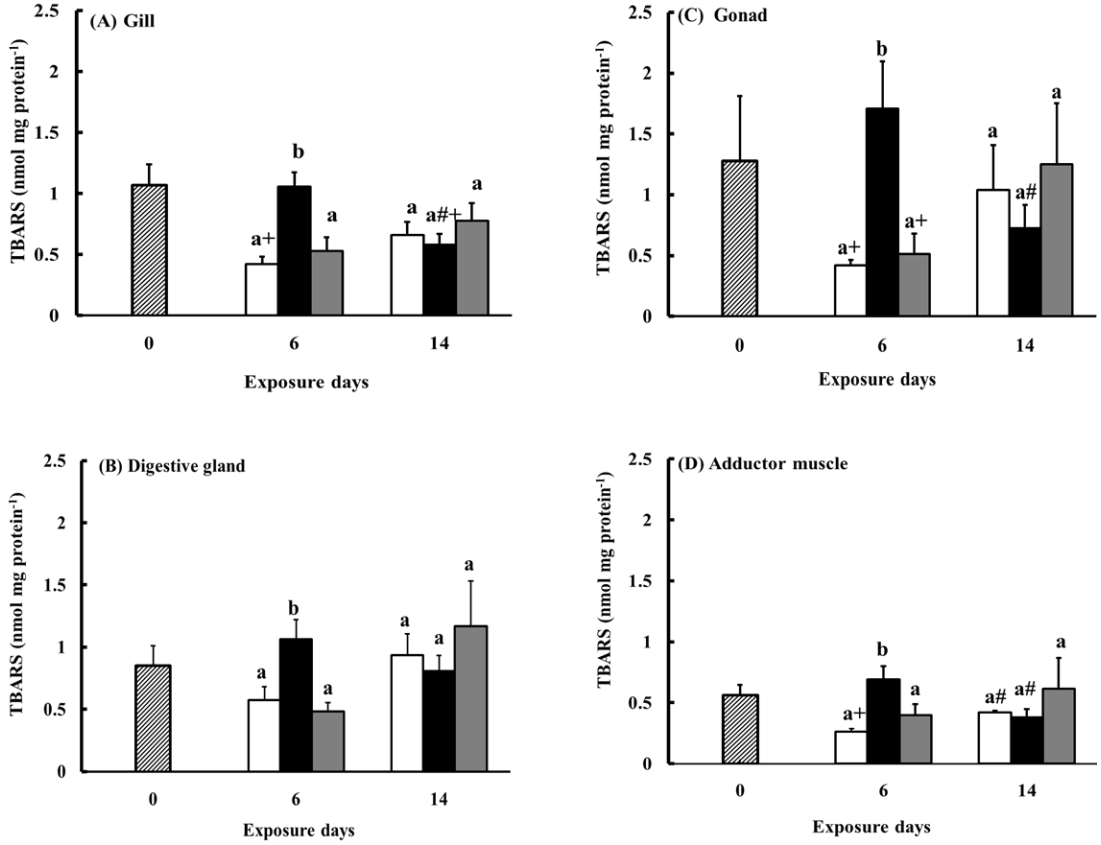


Figure 16: Thiobarbituric acid reactive substances (TBARS) concentration in supernatant of the (A) gill, (B) digestive gland, (C) gonad, and (D) posterior adductor muscle after 14 days exposure to control (no added Hg, white bar) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous (black bar) or intermittent (grey bar) exposure. The hatched bar at time zero are values for unexposed (stock) mussels at the start of the experiment. Data are means ± SEM, nmol mg $protein^{-1}$ for n = 4-6 mussel per treatment at each exposure day. Different letters within exposure day indicates statistically significant treatment effect (ANOVA, $p < 0.05$). # indicates statistically significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents statistically significant time effect compared to day zero (day 0, initial mussel stock) (ANOVA, $p < 0.05$).

4.2.5. Effects of Hg exposure on total glutathione concentration

Tissue total glutathione concentrations were also measured (Figure 17). There was no treatment- dependent effect in the total glutathione concentration in the adductor muscle and the gonad compared to the control or stock mussels (ANOVA, $p > 0.05$). However, treatment-dependent effects (not detectable, < 0.12 nmol GSH g^{-1} ww tissue, Figure 13) in total glutathione concentration were observed in the digestive gland (continuous exposure, day 14) and gills (in both continuous and intermittent exposure) from day 6 up to the end of the experiment (Figure 17). The gonad and adductor muscle showed a transient increase in total glutathione in the continuous and intermittent exposures on day 6 compared to the control, with the rise being greatest in the continuous regime (Figure 17). However, these differences were lost in the adductor muscle and gonad by the end of the experiment (ANOVA, $p > 0.05$, Figure 17). Pearson's linear correlation (*r,* Figure 18) analysis showed a statistically significant negative relationship between tissue Hg and total glutathione concentrations in the gill (-0.70) and digestive gland (-0.37); but not for the gonad (0.03) and adductor muscle gonad (-0.09).

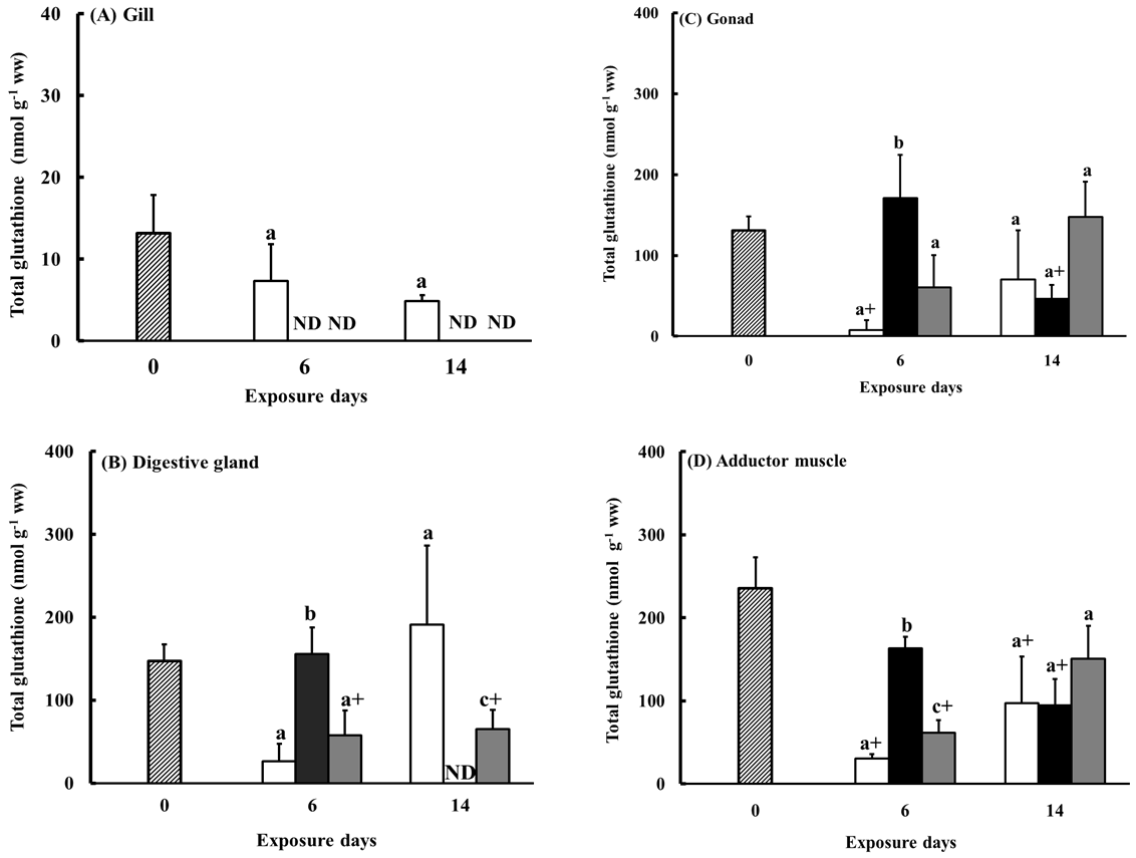


Figure 17: Total glutathione concentration in supernatant of the (A) gill, (B) digestive gland, (C) gonad, and (D) posterior adductor muscle after 14 days exposure to control (no added Hg, white bar) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous (black bar) or intermittent (grey bar) exposure. The hatched bar at time zero are values for unexposed (stock) animals at the start of the experiment. Data are means ± SEM, nmol g^{-1} ww for n = 4-6 mussel per treatment at each exposure day. Different letters within exposure day indicates a statistically significant treatment effect (ANOVA, $p < 0.05$). + represents a statistically significant time effect compared to day zero (day 0, initial mussel stock) (ANOVA, $p < 0.05$). Note ND represent not detected.

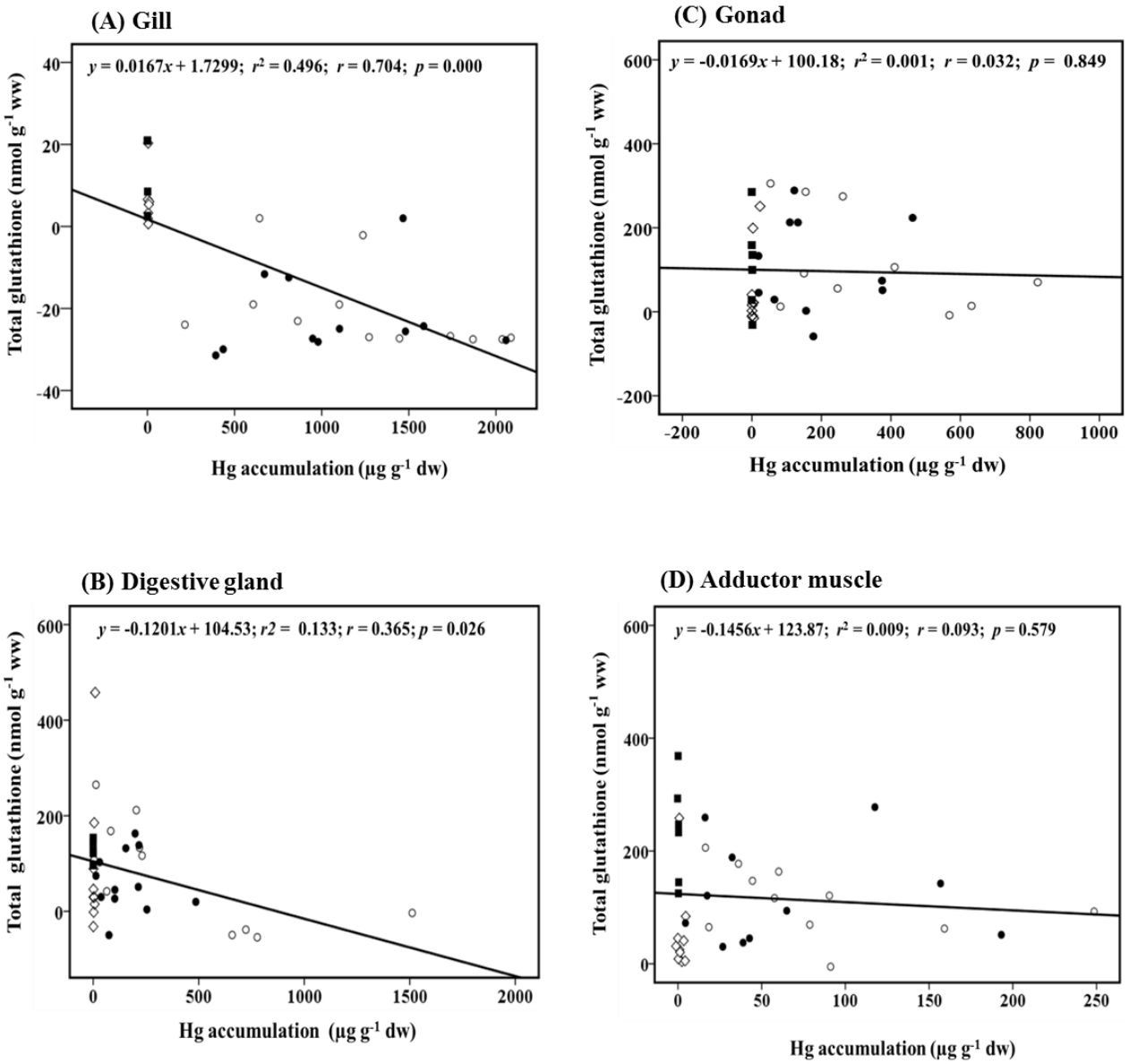


Figure 18: The correlation relationship between the Hg accumulation and total glutathione concentrations of (A) gill, (B) digestive gland, (C) gonad, and (D) posterior adductor muscle after 14 days exposure to control (no added Hg, open diamond) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous (open circle) or intermittent (closed circle) exposure. The black square represents the background Hg concentration in unexposed (stock, day 0) mussels at the start of the experiment. Note: all lines are fitted with a linear model. *r* is the measure of the strength (best fit) of a linear relationship between the two variables. r^2 is the square of *r* and it gives a measure of the amount of variations explained by the model.

4.2.6. Histological Alterations during Hg exposure

The histology of the gill and the digestive gland at the end of the 14 days exposure to 0 (no Hg control) or 50 µg l^{-1} Hg as $HgCl_2$ in continuous or intermittent exposure (Figure 19) were also examined. The gill filaments from the control groups showed some frontal face with cilia (frontal, laterofrontal and lateral); well structure lamella; no evidence of haemocyte infiltration in the long lacuna space or any other pathology (Figure 19A). The gill filaments of mussels from both the continuous and intermittent exposures to Hg showed some gill injuries that were discernible from that of the controls. In the continuous exposure group some mild erosion of the lateral and frontal cilia (6/6 mussels) and desquamation of the gill epithelial cells (3/6 mussels) was observed with evidence of oedema in the epithelium, swelling of the lacuna space, and hyperplasia in the tips of the lamaellae. In contrast to the continuous exposure, the intermittent treatment caused more severe erosion of the tips of the secondary lamellae with hyperplasia of the filaments (6/6 mussels). These observations were confirmed by quantitative histology (Figure 19C). The percentage of gill filaments showing injuries such showing oedema were 0 (not observed), 66, and 0 (not observed) % in the control, continuous and intermittent exposures respectively. The percentages of gill filaments showing hyperplasia were 0 (not observed), 94, and 100% in the control, continuous and intermittent exposures respectively.

The digestive gland of the control mussels exhibited preserved architecture (round or oval) of the digestive tubules and surrounding connective tissues (6/6 mussels). There was no evidence of loss of cilia in the primary ducts ramifying from the stomach, haemocyte infiltration, degeneration and desquamation of the digestive cells or any other damage. The epithelial cells of the digestive tubules were generally normal without any loss of architecture (6/6 mussels, Figure 19D). In contrast, there were treatment-dependent pathologies in the digestive gland of the Hg exposed groups

(continuous or intermittent) compared to the control (ANOVA, $p > 0.05$). For example, Two out of 6 mussels in the continuous exposure showed digestive tubules with desquamation of digestive cells (Figure 19E). Three out of 6 mussels showed loss of cilia in the primary ciliated ducts. Only 1 out of 6 mussels showed slight inflammation, with the mussel showing 26% of the proportional area of the digestive tubules characterised by patches of haemocyte infiltration. One out of 6 mussels showed remnant of waste in the intestine.

All mussels (6/6 mussels examined) in the intermittent exposure showed inflammation characterised by haemocytes infiltration (Figure 19F). Two out of 6 mussels showed granulocytomas characterised by densed aggregation of granulocytes and basophilic cells within the connective tissues. Degeneration characterised by the complete disruption of the digestive tubules due to severe inflammation was observed in 4 out of 6 mussels. Three mussels showed desquamation of digestives cells and 2 showed tubules with necrosis. Also, the height (thickness) of the epithelial cells in the wall of the digestive tubules was statistically significant decreased in the treatment groups compared to the control (ANOVA, $p < 0.05$). However, there were no statistically significant difference in epithelial height thickness between the continuous and the intermittent exposure (ANOVA, $p > 0.05$). Value were (means ± SEM, $n = 6$, µm) 38.3 ± 2.79, 29.2 ±1.43 and 23.8 ± 3.03 for control, continuous and intermittent respectively.

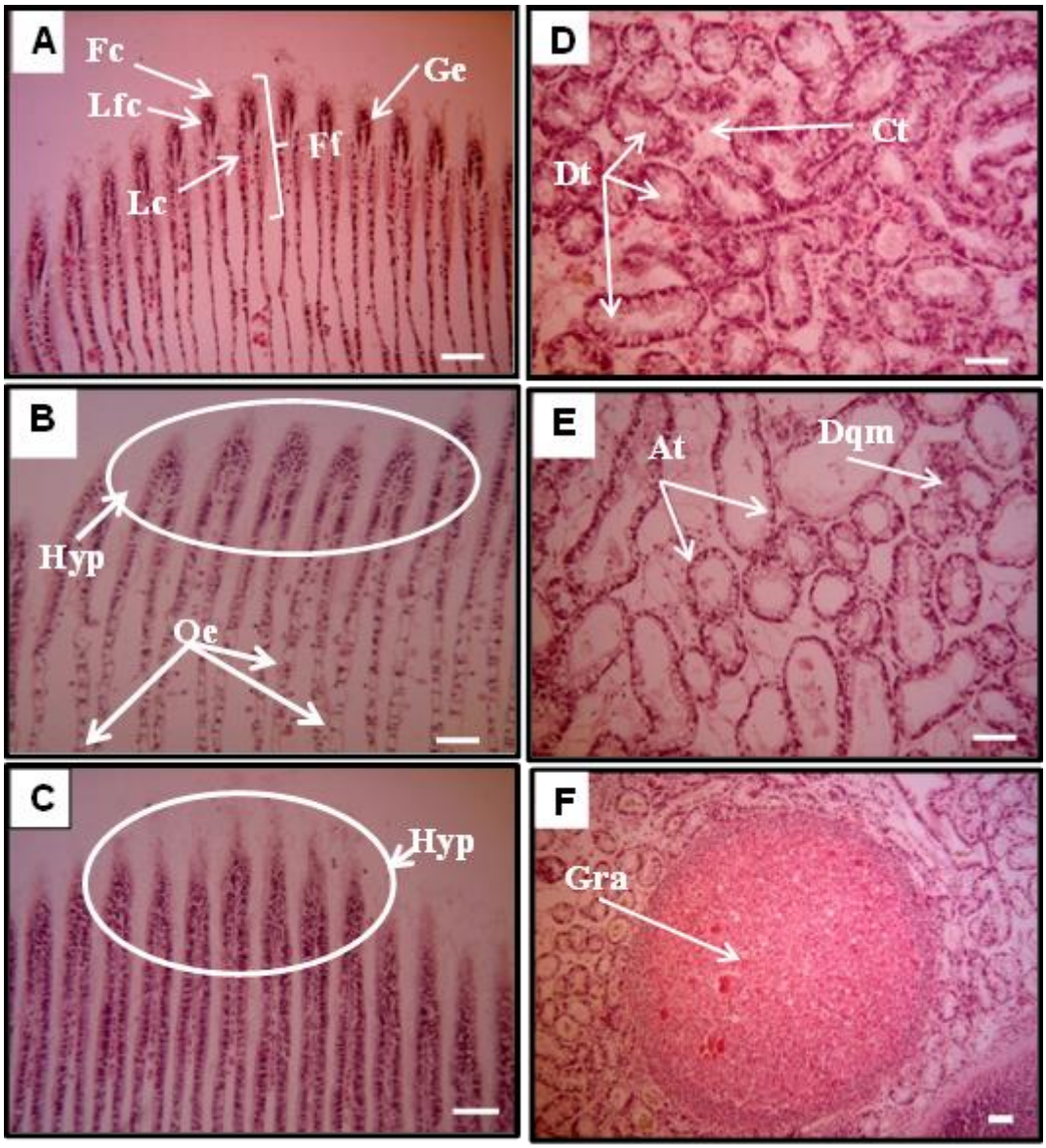


Figure 19: Histology of the gill (A-C) and digestive gland (D-F) of *M.galloprovincialis* after 14 days exposure to control (no added Hg, A, D) or 50 µg Hg l^{-1} as $HgCl_2$ in continuous (B, E) or intermittent (C, F) exposure. Slides were stained with Mayer's Haemotoxylin and eosin. Circle represents gill filaments with hyperplasia (Hyp) on the tips. At = atrophy; Ct = connective tissues; Dt = digestive tubules; Dqm = desquamation; Gra = granulocytomas; Ff = frontal face; Fc = frontal cilia; Lfc = laterofrontal cilia; Lc = lateral cilia; Ge = Gill epithelial; Hyp = Hyperplasia; Oe= oedema; Scale bar: 50 µm.

4. 3. Discussion

The present study provides an overview of the accumulation pattern and biological responses of *M. galloprovincialis* to equal peak concentrations of Hg as $HgCl_2$ in continuous compared to intermittent exposure for up to 14 days. Overall, target organs for Hg accumulation in *M. galloprovincialis* are the same for both the continuous and intermittent exposure regimes, but the time course of Hg accumulation showed some differences between the continuous and intermittent exposures. For example, the gill of the intermittent exposure demonstrated step-wise increases in Hg concentrations corresponding to the exposure profile. Overall, the intermittent exposure group showed less Hg accumulation compared to the continuous counterpart. Despite the differences in Hg accumulation between the continuous and the intermittent groups, some of the biological responses from the exposure were similar by the end of the experiment including haematology (Table 12). However, there were some transient changes in the tissue TBARS and total glutathione concentrations indicating that the Hg exposure was more difficult to manage during the continuous exposure. In contrast the histological examinations made on the gills and digestive gland showed some interesting results. The pathology in the digestive gland of the intermittent exposure was more severe than those of the continuous counterpart. While, the reverse was the case for the gill; pathology as observed in the gill were more severe in the continuous than the intermittent exposures.

4.3.1 Aqueous Exposure to Mercury and Tissue Accumulation

The results of this study have demonstrated that in a sub-lethal exposure concentration of 50 µg Hg l^{-1} as $HgCl_2$ for up to 14 days, *M. galloprovincialis* can accumulate higher levels of Hg during aqueous exposure to either continuous or

intermittent mode compared to control (Figure 15). Background Hg concentration in the control filtered seawater (n = 42) were below detection limit of the ICP-MS (0.28 µg l^{-1} ± 0.05). The nominal 50 µg Hg l^{-1} exposure concentration was confirmed by total measured Hg concentration in the tanks (Figure 14). Daily measurements were also made for pH, dissolved oxygen, salinity and total ammonia in the tanks and there was no statistically significant difference, values were similar in all treatment tanks including the controls. Together these suggest that the seawater quality in the tanks was similar and there were no stress related effects based on the parameters measured.

Our findings indicate similarities in the affinity and distribution of Hg to target organs for both continuous and intermittent exposure with the highest concentrations in the gill > digestive gland > gonad > remaining soft tissue > adductor mussel. The results, supports earlier findings in our laboratory, where greater Hg accumulation was found in the gill and digestive gland, than the adductor muscle after 8 days exposure to 50 µg l^{-1} Hg as $HgCl_2$ (Sheir et al., 2010). The elevated total Hg concentration in the gills compared to the other internal organs is consistent with the waterborne route of exposure, and dissolved Hg uptake by mussels is known to be initially by the gills, then redistributed to the haemolymph and other organs (Roesijadi et al., 1981). It is therefore likely that the low concentrations of Hg seen in the other organs are probably related to Hg transport from the gill. For example, in the continuous exposure of the present study we found around 2 fold increases in gill Hg concentration compared to the digestive gland at the end of the experiment. This phenomena has been previously reported (Roesijadi et al., 1981), they suggested that the gill serves as an uptake site as well as reservoir for the total Hg tissue concentration.

In contrast, the gill of mussels from the intermittent exposure group showed step-wise increases in Hg accumulation, corresponding with the exposure profile (2 day exposure: 2 days in clean seawater alternately, Figure 14). This phenomenon though

has not been previously reported in mussels, is known to occur in fish (Handy, 1995). Handy (1995) exposed rainbow trout and goldfish to a nominal peak concentration of 3 µg Hg l^{-1} $HgCl_2$ for 120h in intermittent compared to continuous mode. He found similar results as the present study, such that there were step-wise increases in Hg accumulation in both fish species examined. Also step-wise observations were made with the whole body burdens in the fathead minnows in the same experiment (Handy, 1995).

In the intermittent exposure, Hg clearance in the gill during the two days in clean water did not match background concentrations; rather a step-wise addition was seen on each exposure day, to the extent that no difference in accumulation between the continuous and the intermittent exposures was seen statistically. The pattern of Hg accumulation in the gills of the intermittent exposure is best explained by the slow clearance of Hg from the tissue, relative to the short time (2 days) in clean water between pulses. Information on the clearance of inorganic Hg accumulated in the gill tissue of *M. galloprovincialis* is lacking. However, clearance of Hg from the gill of oysters has been shown. Denton and Burdon-Jones (1981) recorded the biological half-life ($t_{1/2}$) of Hg in the gill of *Saccostres echinata* to be in the range of 30-47 days after 30 days exposure to 10 µg l^{-1} Hg and 30 day depuration in clean seawater under varying temperature (20-30 ºC) and salinity (20-36 ‰). Also Okazaki and Panietz (1981) reported $t_{1/2}$ of 26 days for total Hg from gills of *Crassostrea gigas* transferred from a polluted site to clean environment. Similarly, estimates of the whole body elimination rate for total Hg from field-collected mussels are between ($t_{1/2}$) 53-293 days (Riisgård et al., 1985). It is therefore also unlikely that the internal organs will clear appreciable amounts of Hg in the two days in clean water used in the present study (net excretion was not observed, Figure 15).

Consequently, the total Hg concentration in the digestive gland, gonad and remaining carcass by the end of the experiment was higher in the continuous compared to intermittent exposure. On the basis of equivalent dose, with half the exposure time, the mussels from the intermittent profile might be expected (in theory) to contain total Hg concentrations that are 50% lower than their continuous exposure counterparts. This theory was not true in the present study. At the end of the experiment the difference between the continuous and the intermittent exposures were 74, 52, 21 % for the digestive gland, gonad, and remaining soft tissue respectively; suggesting that the accumulation hazard is at least broadly consistent with the notion of equivalent dose for the internal organs. However, the gill was more dynamic, and at the end of the exposure the total Hg concentrations were similar between the exposure regimes (Figure 15A). This may relate to the role of the gill in transferring Hg to the internal compartments, or alternatively, it may relate to the gill showing the highest turnover rate of Hg (Denton and Burdon-Jones, 1981).

4.3.2. Effects of Hg Exposure on Haemolymph Chemistry and Ionic Regulation

The immunotoxicity of Hg has previously been reported (Coles et al., 1995; Sheir et al., 2010). Hg causes inflammation which might stimulate the production of circulating haemocytes (Coles et al., 1995; Sheir et al., 2010). In the present study, Hg exposure resulting in both increased and decreased total haemocytes count during continuous and intermittent exposure respectively compared to the control (Table 12). The increase in the total haemocyte count in the continuous exposure might be response from the Hg exposure rather than a reaction to specific challenge (Coles et al., 1995). The decreased in the intermittent regime might relate to the pathology and haemocyte infiltration in the tissue (e.g., digestive gland). Bivalves have open circulatory system,

and there is the possibility of tissue cells migrating to the circulatory system and vice versa (Auffret and Oubella, 1994). The phenomenon where immunoactive haemocytes in the haemolymph migrated and mobilised in tissues where injury are more likely to occur has been described in bivalve under stress conditions (Seiler and Morse, 1988; Marigómez et al., 1990).

The neutral red retention (NRR) ability of the haemocyte was also measured in our study (Table 12). NRR is based on the ability of viable cells to incorporate and bind neutral red dye in the lysosome and has proven to be an effective tool in the elucidation of cytotoxicity and an indicator of general cell health (Lowe et al., 1995). The neutral red retention of the haemocyte in our study was not affected by Hg treatment (Table 12), indicating that the immunological functions of the haemocytes were still intact up to the end of the experiment. The result is consistent with previous studies (Sheir et al., 2010), who also reported no treatment effect on NRR in *M.edulis* after 11 days continuous exposure to 50 µg Hg l^{-1} as $HgCl_2$, whilst also showing that the cells were capable of infiltrating the digestive gland as part of the normal inflammatory response. Plasma glucose concentrations were relatively low in all treatments including the control. This is expected, mussels were starved throughout the exposure period (Sheir et al., 2010).

Despite the difference between the continuous and the intermittent exposure on the tissue Hg accumulation, there were no major differences in plasma or tissue electrolyte concentrations. Plasma Na^+, K^+ and osmotic pressure showed some transient treatment or time effect (Table 12), which were lost by the end of the experiment. Values for electrolytes in the haemolymph remained in the normal range for *M.edulis* (Potts, 1954; Sheir et al., 2010), with no treatment-related effects (Table 12). Also, the tissue electrolytes (Na^+, K^+, Ca^{2+} and Mg^{2+}, Tables 13-16) examined in the gill, digestive gland, gonad, remaining soft tissue and adductor muscle did not show any

difference in between treatments, suggesting that the osmoregulatory ability of the mussels was not disturbed by either the continuous or intermittent exposure.

4.3.3. Oxidative Stress and Organ Pathology during Hg Exposure

Metal accumulation in tissues provides an important link to adverse effects on the physiological and histological examinations in mussels (Al-Subiai et al., 2011). Mercury exposure has been shown to cause lipid peroxidation assessed oxidative damage in mussels (Garron et al., 2005). In the present study, there was no difference in TBARS concentration in the intermittent compared to the continuous exposure at the end of the experiment. However, there was a transient increase in TBARS concentration of the supernatant from all tissue (gill, digestive gland, gonad and adductor muscle) examined on day 6 in the continuous compared to the intermittent or control (Figure 16). This suggests that mussels faced oxidative challenge (day 6) but were able to counteract the pressure from the Hg exposure by the end of the experiment. Values of TBARS in Hg treatment at the end of the experiment were not significantly different from the controls (Figure 16). However, at a lower total Hg concentration of 20 µg Hg l^{-1} *in M.edulis* exposed for 21 days, Géret et al. (2002) observed increased malonialdehyde (MDA) concentration in the gills and digestive gland.

The total glutathione concentration was also measured in the gill, digestive gland, gonads and adductor muscle (Figure 17). There was no evidence of glutathione depletion in the adductor muscle or gonad from any treatment by the end of the experiment. However, total glutathione concentrations in the gills of mussels from both Hg exposure regimes were below the detection limit (< 0.12 nmol GSH g^{-1} ww tissue) from day 6 up to the end of the experiment. The digestive gland also showed below detection of total glutathione concentrations in the continuous exposure at day 14

(Figure 17). As explained earlier (Section 3.4), Hg interfered with the assay, and is evident as the rate of change of absorbance in treated group (e.g., gills) was less than the blank giving rise to apparent negativity. The glutathione response in the gill was tissue Hg concentration-dependent with an inverse relationship between total GSH and Hg accumulation, regardless of the exposure regime used (Pearson's r = - 0.7, p = 0.001). There are several possible explanations for this effect: (i) Hg accumulation causes oxidative stress and the total glutathione pool is genuinely depleted, (ii) the tissues become leaky during Hg exposures and glutathione is lost from the cell due to membrane damage, (iii) Hg complexes rapidly with reduced GSH making glutathoine unavailable to the cell, and in supernatants also unavailable to react in the GSH assay or (iv) Hg directly interferes with the enzyme, glutathione reductase by inhibiting the reduction of GSSH to GSH hence hindering subsequent recycling of the GSH. The first option seems unlikely since TBARS did not increase in the gills. Some membrane leak is possible given the presence of injury to the gill epithelium in both the continuous and intermittent exposures (Figure 19); and the oedema observed suggests some changes in water permeability. However, Hg complexation with reduced GSH seems likely. Mercury has a high affinity for sulfhydryl-containing molecules or thiols such as reduced glutathione GSH, metallothionein, and cysteine (Rabestein et al., 1985). In the present study, we therefore interpret at least some of the decline in the total GSH in the gill and digestive gland as a loss of bioavailable GSH. It is also possible that Hg^{2+} ions could inhibit glutathione reductase in the tissue (or in the GSH assay) to prevent the reduction of the GSSH to GSH. Spiked test showed that additions of Hg did cause lower total GSH concentrations, and either of the two latter mechanisms (GSH binding, or glutathione reductase inhibition) might cause this result. In the main experiment, the rate of change of absorbance in treated group (e.g., gills) was less than the blank giving rise to apparent negative glutathione concentrations, which can only be explained if

glutathione reductase is inhibited. Interestingly, the glutathione response in the gill was tissue Hg concentration–dependent with an inverse relationship between total GSH and Hg accumulation, regardless of the exposure regime used (Pearson's r = -0.7, p = 0.001) suggesting a progressive apparent loss of GSH activity or bioavailability. However, in contrast, Canesi et al. (1999) showed no statistically significant effect on total glutathione concentration in the gill and digestive gland of *M. galloprovincialis* exposed to 0.2 µmol l^{-1} (40 µg l^{-1}) Hg as $HgCl_2$ for up to 7 days. This difference may be due to the lower total Hg concentration, the shorter exposure time used by Canesi et al. (1999), although methylmercury exposure did cause decreases in total glutathione concentration in both gill and digestive gland (Canesi et al., 1999).

Up to 14 days exposure to Hg (50 µg Hg l^{-1} as $HgCl_2$) to either continuous or intermittent mode resulted in histological changes in the gills and digestive gland of *M. galloprovincialis.* Hg exposure has been shown to result in mild gill injury such as hyperplasia, cilia (frontal, laterofrontal and lateral) erosion (Sheir et al., 2010). In the present study the gill of both continuous and intermittent exposure to Hg showed hyperplasia (Sheir et al., 2010).

Hg exposure to either continuous or intermittent mode resulted in pathology of the digestive gland (Figure 19). Both exposure modes showed inflammation, degeneration (complete disruption of the tubule architecture) of the epithelial and desquamation (shedding) of the digestive cells. However, the pathology seen in the intermittent exposure were more severe compared to the continuous exposure. Notably, in the intermittent exposure, four out of six (4/6) mussels showed desquamation of the tubules epithelia cells, all the six (6/6) mussels showed haemocyte infiltration and granulocytomas (dense aggregation of haemocyte) was seen in two out of the six mussels (2/6). The latter was not observed in controls or the continuous exposure to Hg. The biological importance of the granulocytomas in only two mussels is unclear,

and it may be chance that these were not observed in the continuous exposure group. Desquamation of the digestive cells in the present study suggests that the cells were target. For the continuous exposure only one out of the six mussels that were examined showed slight inflammation. The observations here suggest the inflammation due to Hg may be more important in an intermittent profile especially in the digestive gland.

4.3.4. Conclusions and Implications in Risk Assessment

In conclusion, the equal peak concentrations concept adapted in the present study has demonstrated that Hg accumulation in the intermittent is generally less than the continuous exposure but that in some aspects of physiological responses the intermittent exposure can be more severe than expected. In terms of risk assessment, the findings support the notion that the standard ecotoxicity test may underestimate the toxicity of intermittent exposure to Hg at least for *M. galloprovincialis*. For environmental monitoring programmes, such as Gulfwatch (Chase et al., 2001), this study shows that some sub-lethal indicators can be correlated with mercury exposure, and therefore may be more useful than incidence of mortality in making management decisions on the protection of the marine environment, as well as giving an organism health context to measurements made on the metal accumulation by mussels.

Chapter 5 *:*

Accumulation and sub-lethal physiological effects on the blue mussel, Mytilus galloprovincialis, during continuous exposure to a mercury and cadmium mixture

Abstract

Mussels were exposed using a semi-static and triplicated design to either control (no added metal), 50 µg l^{-1} (Hg alone), 50 µg l^{-1} (Cd alone) and 50 µg l^{-1} Hg plus 50 µg l^{-1} Cd mixture for 14 days. Tissues were collected on days 0, 2, 4, 8 and 14 for metal analysis and sub-lethal responses using a suite of assays. Tissues metal concentration was not significantly different in the single metal (Hg or Cd) compared to the Hg plus Cd mixture treatment for all tissues, apart from the gill of the Cd alone treatment. At the end of the experiment the gill Cd concentration was significantly increased in the Hg plus Cd mixture compared to the Cd alone treatment suggesting the influence of Hg on Cd uptake. Comparison of the arithmetic sum total of the individual metals to the actual values of the Hg plus Cd mixture treatment statistically resulted in no interaction (additive) in accumulation in all the tissues examined. There were no observed treatment effects on total haemocyte count, haemolymph protein, or glucose concentration in the cell-free haemolymph. Neither was there any treatment effect on the osmotic pressure, ions in the tissues or in the cell-free haemolymph. At the end of the experiment, Hg-mediated oxidative damage as a result of increased thiobarbituric reactive substances (TBARS) and apparent depletion of total glutathione was seen in the gill and digestive gland of the Hg alone and Hg plus Cd mixture. Histopathology examination showed similar pathology in the Hg alone, and the Hg plus Cd treatment. In conclusion, continuous exposure to a mixture of Hg plus Cd resulted in an additive effect in accumulation and all sub-lethal endpoints measured were not affected apart from the tissue TBARS, hence in terms of risk assessment the set regulation for the individual metals will protect for the mixtures of Hg plus Cd at least for adult *M. galloprovincialis*.

5.0. Introduction

In the aquatic environment, mussels are typically confronted with mixtures of contaminants rather than in isolation to an individual contaminant, concurrently or sequentially (Altenburger et al., 2013). Exposure to these mixtures of contaminants, e.g. metals, can result in biological responses which may be different from the effects of the individual contaminant (Altenburger et al., 2003). The combined effects of these mixtures can be in several forms; potentiation, where a chemical is not toxic by itself, but enhances the toxicity of another in a mixture; additive or non-interaction effect, where the toxicity of the combination acts without diminishing or enhancing each other effect when compared to the toxicity of the individual metals; where the toxicity of the combination is larger or smaller than the sum of the individual metals, there is synergistic or antagonistic effect respectively (Altenburger et al., 2003; Kortenkamp and Hass, 2009).

A number of studies have demonstrated the sub-lethal effects of Hg (Abel, 1976; Strömgren, 1982; Roesijadi et al., 1984; Micallef and Tyler, 1990; Viarengo et al., 1994; Canesi et al., 1999; Fernandez and Beiras, 2000; Géret et al., 2002; Yap et al., 2004; Pattnaik et al., 2007; Sheir et al., 2010) or Cd (Redpath and Davenport 1988; Gardner, 1993; Coles et al, 1995; Fernandez and Beiras, 2000; Tran et al., 2007; Sheir and Handy 2010; Amachree et al., 2013) to aquatic organisms. However, only a few studies have addressed the combined effects of metals to *Mytilus species*. Binary mixtures of Hg plus Cd resulted in synergistic effects on the survival, filtration rate, and oxygen consumption of *Perna viridis* (Mohan et al., 1986). Hg plus Cu produced an antagonistic effect of Cu on Hg accumulation in the digestive gland (Raftopoulou and Dimitriadis, 2011) in *M. galloprovincialis.* A tertiary mixture of Hg plus Cd plus Cu produced antagonistic response on embryogenesis of *M. galloprovincialis* (Prato and Biandolino, 2007). Hg and Cd occur together in the earth's crust associated with zinc

and copper ores, and may be co-released into the aquatic environment in effluents from mining and smelting operations, coal combustion and other industrial sources especially those associated with the refining of ferrous and non-ferrous metals and waste incinerators (Nriagu, 1989; Cheng et al., 2014; UNEP, 2013). Thus, the study of the combined effect (interactions) will provide a more realistic assessment of their toxicity rather than observation from the individual metals.

The present study was designed to assess the interactive toxicity of Hg plus Cd using the observed toxicity of the individual metals as a control in the same experiment. The hypothesis is that the arithmetic sum total of the individual metals will be equal to the sum total of the combinations, thus producing an additive effect. The method of assessing the interactive toxicity of the binary metal combination (Hg plus Cd) involved testing the single metals as well as the combination using an equal concentration of each metal. To assess the combined effects, tissue metal accumulation and a range of sub-lethal endpoints relating to the main physiological processes were measured. These included osmoregulation (tissue electrolytes, cell-free haemolymph electrolytes and osmotic pressure), oxidative stress parameters (lysosomal membrane damage via neutral red retention, total glutathione and thiobarbituric acid reactive substances, TBARS), and general animal health (haematology and organ pathology).

5.1. Methodology

5.1.1. Test Organisms and Acclimation

Mussels (shell length: 40-60 mm) were collected in November, 2011 from Port Quin and acclimated for two weeks as described (Sections 2.2). Water Quality parameters were measured daily in the stock filtered seawater and changed twice weekly during the acclimation period.

5.1.2. Experimental Design

One hundred and thirty-two mussels (means ± SEM, whole weight, 22.5 ± 0.5 g; shell length, 55.5 ± 0.4 mm) were randomly selected from the stock and allocated to twelve (12) glass aquaria containing 10 l of filtered seawater. All glass aquaria were acid washed and dried prior to use (Section 2.3). A total of 11 mussels were allocated to each glass aquaria containing 10 l of filtered seawater. Mussels were not fed 24 h prior to transferring to experimental tanks or during the exposure periods.

After 24 h acclimation to the experimental tanks, one mussel from each of 12 experimental tanks (a total of 12 mussels) were collected on day 0 (stock mussels, not exposed) for reference measurements on the mussels. Six mussels out of the 12 were used for trace elements analysis, cell-free haemolymph ions, osmotic pressure and the remaining 6 mussels used for total haemocyte counts, cell-free haemolymph glucose, tissue total glutathione, and tissue TBARS concentration on day 0. The remaining mussels (10 mussels/tanks; 30 mussels/treatment) were exposed in a triplicate design to four treatments including the controls in a semi-static exposure regime. The treatments were; (a) control, no added Hg or Cd; (b) 50 µg l^{-1} Hg alone as $HgCl_2$; (c) 50 µg l^{-1} Cd alone as $CdCl_2$; or (d) 50 µg l^{-1} each of Hg plus Cd (mixture exposure, Hg plus Cd) during continuous exposures. Test media were changed and re-dosed daily. Dosing

was achieved by adding 0.5 ml of 1 g l^{-1} Hg and/or Cd stock solution to tanks containing 10 l of seawater.

The quality of the test water (pH, salinity, dissolved oxygen, total ammonia) was analysed daily as described previously (Chapter 2). The background metal concentrations in the control filtered seawater (means, *n* = 42, Figure 20) were below the detection limit of the instrument (Hg, 0.27 ± 0.1 and Cd, 0.11 ± 0.1 µg l^{-1}). The electrolyte composition of the seawater was also analysed (means ± SEM, *n* = 50, mmol l^{-1}); Na^{+}, 456 ± 2.1; K^{+}, 9.2 ± 0.1; Ca^{2+}, 12.2 ± 0.1; Mg^{2+}, 67.1 ± 0.4. Mussels (2/tanks; 6/treatment) were randomly collected at day 2, 4, 8 and 14 and analysed for tissue trace elements and all endpoints described (Section 2.3) but without the neutral red retention for logistic reasons.

5.1.3. Haemolymph Extraction and Tissue Collection

Mussels were collected from glass aquaria on sampling days (2, 4, 8 and 14) rinsed to removed excess Hg and/or Cd. Haemolymph and tissues (adductor muscle, digestive gland, gill, gonad, and remaining soft tissue) were collected and stored exactly as described in Chapter 2, Section 2.4.

5.1.4. Trace Metal Analysis

The tissues and seawater were analysed using ICP-OES and ICP-MS respectively, for Hg and/or Cd concentration and electrolytes compositions. The procedural detection limit of the instrument for seawater (ICP-MS, means ± SEM, *n* = 6, µg l^{-1}) were 0.27 ± 0.1 and 0.11 ± 0.1 for Hg and Cd respectively. While those of the tissue digest on the ICP-OES were 0.24 ± 0.1 and 0.02 ± 0.1 µg l^{-1} for Hg and Cd

respectively. For a typical 0.1 g of tissue the detection limit equates to 2.4 µg Hg g^{-1} dry weight and 0.2 µg Cd g^{-1} dry weights for Hg and Cd respectively. Calibrations on the ICP-OES and ICP-MS were performed as described in Chapter 2, Section 2.5.

5.1.5. Biochemistry and Haematology

Tissues were homogenised (Cat X520D homogeniser with a T6 shaft, medium speed, Bennett and Company, Weston-super-Mare) in 5 volumes of ice-cold buffer as described (Section 2.7.1). Total glutathione (i.e., reduced GSH and oxidised GSSH) concentration of the tissue was determined based on the enzymatic recycling method as described (Section 2.7.3). The tissue TBARS concentration was determined as described (Section 2.7.3). Tissue supernatant protein concentrations were determined as described (Section 2.8). Total haemocyte of the haemolymph was counted as described (Section 2.10). The osmotic pressure, ions and glucose concentration were determined in the cell-free haemolymph as described (Section 2.11 and 2.12).

5.1.6. Calculation and Statistical Analysis

All statistical analyses were performed using StatGraphic Plus for windows version 5.1 as described in Chapter 2, Section 2.13. In addition, the concept of the toxic unit (Sprague, 1970), was used to express the interactive effects of the binary mixtures. If the effect of the binary combination is additive, then the simple arithmetic sum of the measured values of the two chemicals would be equal to the measured value of the binary combination. In cases where the measured value of the binary combination is lesser or greater than the sum of the individual chemicals, the interaction is antagonistic or synergistic respectively (Equation 1). Interactive effects are considered if statistically

significant differences exist between the sum of the individual metals and their combination at a 95 % confidence level by the Student's t-test.

A + B = C----------Additive

A + B = > C----------Synergistic

A + B = < C----------Antagonistic

Where ''A'' and ''B'' are the measured values of the individual metals and ''C'' the measured value of the combination.

5.2. Results

5.2.1. Aqueous Exposure to a Mixture of Hg and Cd

Aqueous exposure to Hg and Cd singly or in combination did not result in mortality during the course of the experiment, confirming that a sub-lethal exposure was conducted. The background concentrations of Hg and Cd in the control filtered water (means, $n = 42$, µg l^{-1}, Figure 20) were below the detection limit of the instrument (0.23 ± 0.03 and 0.11 ± 0.01 for Hg and Cd respectively). The 50 µg l^{-1} nominal concentration for metals (Hg and/or Cd) concentration in the seawater was confirmed by the measured concentrations from the tanks (Figure 20). There were no statistically significant difference on Hg concentration between the Hg alone and the Hg plus Cd mixture (Student's t-test, $p = 0.29$). Neither were there significant differences between the Cd alone and the Hg plus Cd treatment for Cd concentration (Student's t-test, $p = 0.05$). The target water concentrations was achieved and values (means ± SEM) were 47.3 ± 1.8, 54.8 ± 2.1, 48.0 ± 1.3, 51.8 ± 0.9 µg l^{-1} for Hg alone, Cd alone, Hg (Hg plus Cd) and Cd (Hg plus Cd) mixture respectively.

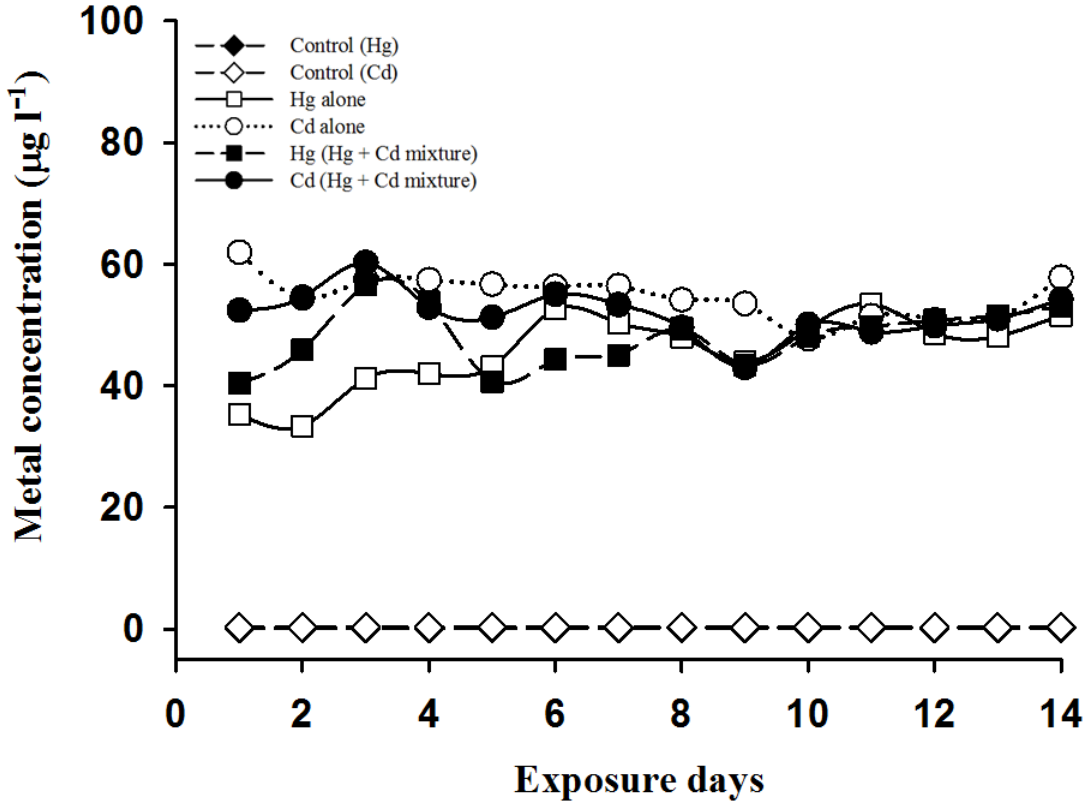


Figure 20: Mercury and cadmium concentration in seawater after 14 days continuous exposure to control [no added Hg (closed diamond) or Cd (open diamond) on dashed line], 50 µg l^{-1} Hg alone (open squares on solid line), 50 µg l^{-1} Cd alone (open circle on dotted line), or 50 µg l^{-1} Hg (closed squares on dashed line) plus 50 µg l^{-1} Cd (closed circle on solid line) mixture. Water samples were collected immediately after the daily renewal of the test media. Data are means, µg l^{-1} for n = 3 tanks per treatment at each exposure day. Error bars were not shown for clarity.

5.2.2. Tissue Metals Accumulation

Fourteen days of exposure to Hg or Cd singly, or in combination, to *M.galloprovincialis* showed a statistically significant treatment-dependent increase in the relevant metal concentrations in all tissues examined, compared to the unexposed control or stock mussels (Figures 21 and 22 for Hg and Cd respectively, ANOVA, $p < 0.05$). Two-way ANOVA showed statistically significant treatment and time interactions (2-way ANOVA, $p < 0.001$) in both Hg and Cd accumulation for all tissues examined apart from the gonad. The gonad showed significant (ANOVA, $p = 0.03$) and not significant (ANOVA, $p = 0.3$) interactions for Hg and Cd respectively.

There was no statistically significant treatment-dependent differences in Hg accumulation between the Hg alone treatment and the Hg plus Cd mixture treatment in all the tissues examined; apart from a transient increase on day 4 in the gonad which was lost by the end of the experiment (Figure 21, ANOVA $p > 0.05$). All tissues examined showed a statistically significant time-dependent increase of tissue Hg concentration compared to the initial stock mussel (day 0, ANOVA, $p < 0.001$). An increase of Hg accumulation was observed in the Hg alone and the Hg plus Cd mixture treatments, which was statistically significant at some time point. For example, both the adductor muscle and haemolymph showed a significant increase in Hg accumulation from day 8 up to end of the experiment in the Hg and Cd mixture treatment (ANOVA, $p < 0.05$, Figure 21), but not for the Hg alone treatment.

Similar to Hg accumulation, there was no overall treatment-dependent differences in Cd accumulation between the Cd alone and the Hg plus Cd mixture treatment for all tissues examined, except the gill (Figure 22, ANOVA, $p > 0.05$). Cd concentration in the gills was significantly decreased in the Cd alone treatment compared to the Hg plus Cd mixture treatment from day 8 up to the end of the experiment (Figure 22, ANOVA, $p = 0.001$). A transient decrease in Cd concentration

in the Cd alone group compared to the Hg plus Cd mixture treatment was also observed in the gonad and remaining soft tissue, but this effect was lost by the end of the experiment. All tissues examined showed a statistically significant time-dependent tissue Cd concentration compared to the initial stock mussel on day 0 (ANOVA, $p < 0.001$). The Cd alone group showed some transient significant increase in tissue Cd concentration over time. However, a statistically significant increase in the Cd accumulation was observed in the Hg plus Cd mixture treatment for the gill and digestive gland at the end of the experiment compared to the previous time point (ANOVA, $p < 0.05$).

Exposure to a combination of Hg plus Cd showed non-interactive effects of the metals in all the tissues examined (Figure 23). The binary combination resulted in increased metal accumulation in the Hg plus Cd mixtures compared to the arithmatic sum of the individual metals for all tissues (Figure 23). The percentage increases of the Hg plus Cd combination compared to the sum of the individual metals were (%) 20.2, 9.3, 25.1, 23.8, 10.7, and 12.4 for adductor muscle, digestive gland, gill, gonad, remaining soft tissue and haemolymph respectively. However, there were no statistically significant differences between the sum of the individual metal and those of the mixtures (Student's t-test, $p > 0.05$) suggesting additive effect for tissue metal accumulation.

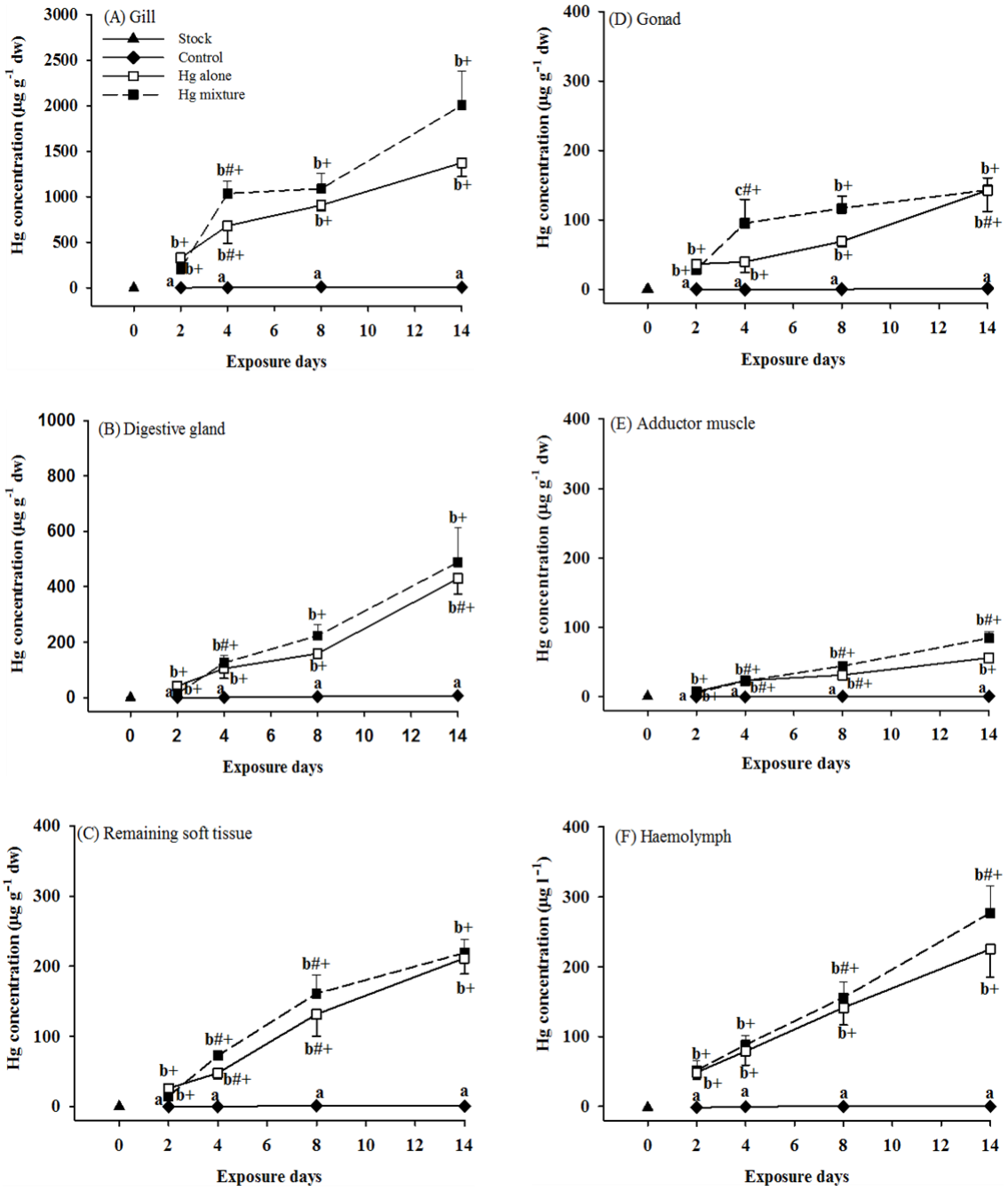


Figure 21: Mercury concentrations in the (A) gill, (B) digestive gland, (C) remaining soft tissue, (D) gonad, (E) posterior adductor muscle and (F) haemolymph after 14 days continuous exposure to control (no added Hg, diamonds on solid line), 50 µg l^{-1} Hg alone (open squares on solid lines) or 50 µg l^{-1} each for the Hg plus Cd mixture (closed squares on dashed lines). The black triangle at time zero represents the background Hg concentration in the unexposed (initial) mussels at the start of the experiment. Data are means ± SEM, µg Hg g^{-1} dry weight tissue, n = 4-6 mussel per treatment at each exposure day. Different letters within the exposure day indicates a significant treatment effect (ANOVA, $p < 0.05$). # indicates a significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, initial mussel stock) (ANOVA, $p < 0.05$).

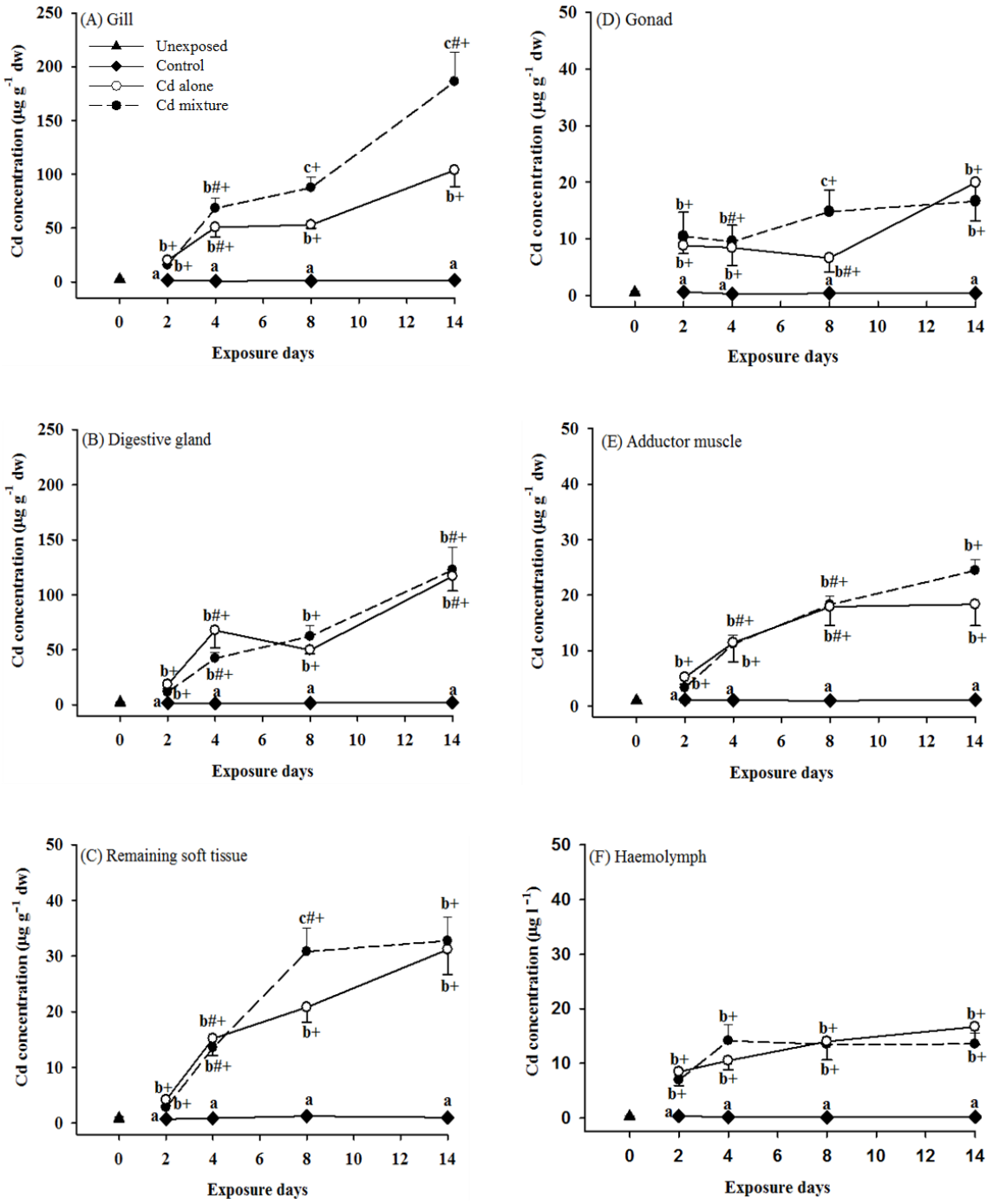


Figure 22: Cadmium concentrations in the (A) gill, (B) digestive gland, (C) remaining soft tissue, (D) gonad, (E) posterior adductor muscle and (F) haemolymph after 14 days continuous exposure to control (no added Cd, diamonds on solid line), 50 µg l^{-1} Cd alone (open cycles on solid lines) or 50 µg l^{-1} each for the Hg plus Cd mixture (closed cycles on dashed lines). The black triangle at time zero represents the background Hg concentration in the unexposed (initial) mussels at the start of the experiment. Data are means ± SEM, µg Cd g^{-1} dry weight tissue, n = 4-6 mussel per treatment at each exposure day. Different letters within the exposure day indicates a significant treatment effect (ANOVA, $p < 0.05$). # indicates a significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, initial mussel stock) (ANOVA, $p < 0.05$).

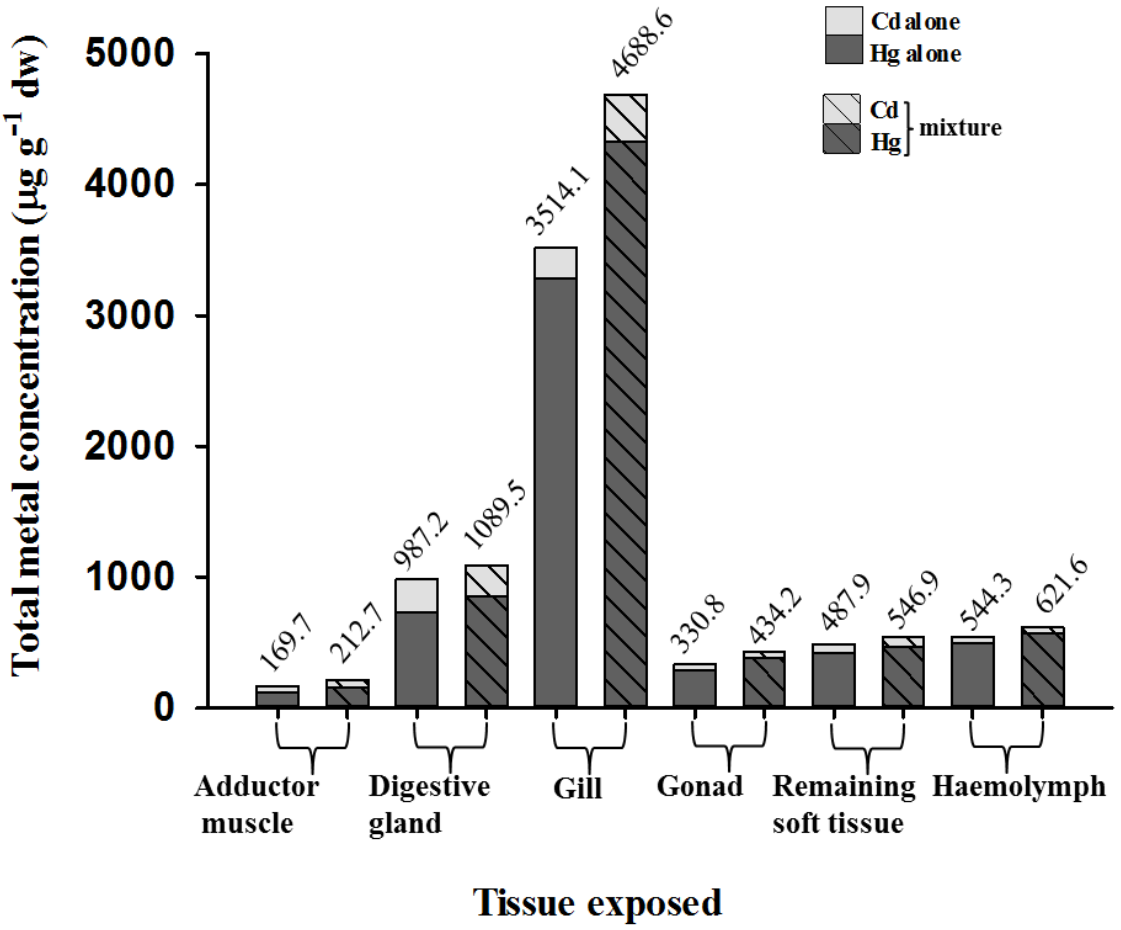


Figure 23: Tissue total metal concentration after 14 days continuous exposure to individual (Hg or Cd) or mixture (Hg plus Cd mixture). The bars without pattern fill represent the individual metals (Hg, grey and Cd, white). The bars with diagonal pattern fill represent a combination of metals (Hg, grey and Cd, white). The value on each bar represents the arithmetic sum of the metals accumulated in each of the tissue examined.

5.2.3. Effects of Hg plus Cd Mixture on the Haemolymph Chemistry and Ion Regulation

Total haemocyte count (THC), whole haemolymph protein and glucose concentration in the cell-free haemolymph were measured at the start and the end of the experiment. There was no statistically significant treatment-dependent differences in the total haemocyte count or whole haemolymph protein (ANOVA, $p > 0.05$, Table 17). Neither was there any significant treatment-dependent effect in the glucose concentration of the cell-free haemolymph (ANOVA, $p > 0.05$, Table 17) in all the treatments including controls. Values (means, $n = 6$) range from 1.9 to 4.8 $\times 10^6$ cells ml^{-1}; 0.7 to 1.1 mg ml^{-1} and 0.1 to 0.2 mmol l^{-1} for total haemocyte counts, whole haemolymph protein and glucose respectively. However, all the parameters measured showed a statistically significant time effect in all treatments at the end of the experiment (day 14) compared to the initial stock mussel (ANOVA, $p < 0.05$, Table 17).

There was no overall treatment-dependent effect on the osmotic pressure compared to the controls except on day 14. On day 14, mussels exposed singly or in combination to Hg + Cd showed a significant decrease in the osmotic pressure compared to the controls (ANOVA, $p = 0.005$, Table 18). There was no significant difference between the Hg alone, Cd alone or the Hg plus Cd mixture treatment. Some time-dependent differences in the osmotic pressure was observed in all treatments, apart from the control (ANOVA, $p < 0.05$, Table 18).

There were no overall statistically significant treatment-dependent effects in the Na^+ and K^+ concentration of the cell-free haemolymph (ANOVA, $p > 0.05$, Table 18). However, there was a significant decrease by the end of the experiment in the Na^+ concentration in the Cd alone and the Hg and Cd mixture treatment. There were some time-dependent significant increases (Na^+) or decreases (K^+) in the electrolytes concentrations compared to the initial stock mussel (ANOVA, $p < 0.05$). Overall, the

concentrations of Na^+, K^+ and osmotic pressure throughout the experiment remained within the normal range (991-1010 mOsm Kg^{-1}, 417-522 mmol l^{-1} and 6-7 mmol l^{-1}) for *M. galloprovincialis.*

Table 17: Total haemocyte counts (THC), whole haemolymph protein and glucose concentration in the cell-free haemolymph from *M.galloprovincialis* after 14 days continuous exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$, and 50µg l^{-1} each for the Hg plus Cd mixture.

Parameters	Treatments	Exposure days	
		0	14
THC (10^6 cells ml^{-1})	No metal control	4.77 ± 8.70	2.55 ± 3.91a+
	Hg alone	-	2.83 ± 3.02a+
	Cd alone	-	1.90 ± 5.09a+
	Hg + Cd mixture	-	2.40 ± 2.87a+
Haemolymph Protein (mg ml^{-1})	No metal control	1.07 ± 0.10	0.82 ± 0.12a
	Hg alone	-	0.92 ± 0.17a
	Cd alone	-	0.65 ± 0.02a+
	Hg + Cd mixture	-	0.74 ± 0.07a+
Glucose (mmol l^{-1})	No metal control	0.07 ± 0.03	0.12 ± 0.04a+
	Hg alone	-	0.21 ± 0.05a+
	Cd alone	-	0.11 ± 0.03a+
	Hg + Cd mixture	-	0.20 ± 0.02a+

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). + represents significant time effect compared to time zero (day 0, stock mussels, ANOVA, $p < 0.05$).

Table 18: Osmotic pressure, Na^+ and K^+ concentration in the cell-free haemolymph from *M.galloprovincialis* after 14 days continuous exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture.

Parameters	Treatments	Exposure days				
		0	2	4	8	14
Osmotic Pressure (mOsm kg^{-1})	No metal control	995.8 ± 2.9	998.3 ± 9.1a	994.8 ± 2.2a	1010.3 ± 5.1a	1003.6 ± 2.6a
	Hg alone	-	1000.2 ± 3.5a	1005.5 ± 4.6a+	1000.7 ± 2.2a	987.8 ± 1.8b#+
	Cd alone	-	1003.7 ± 4.0a	1000.6 ± 3.5a	1001.8 ± 1.7a	993.2 ± 3.6b
	Hg + Cd mixture	-	1010 ± 4.6a+	1005 ± 4.3a	1009.3 ± 4.5a	991.3 ± 1.9b#
Na^+ (mmol l^{-1})	No metal control	417.2 ± 5.3	429.9 ± 7.7a	465.8 ± 6.6a#+	466.2 ± 7.8a+	490.9 ± 1.7ab+
	Hg alone	-	431. 1 ± 9.9a	481.8 ± 22.7a#+	487.4 ± 13.1a+	522.0 ± 22.3a+
	Cd alone	-	497.9 ± 7.5b+	505.5 ± 13.2a+	459.9 ± 8.4a#+	454.8 ± 17.2b+
	Hg + Cd mixture	-	475.4 ± 5.2b+	483.2 ± 7.5a+	456.7 ± 7.2a#+	453.5 ± 6.1b+
K^+ (mmol l^{-1})	No metal control	7.2 ± 0.3	7.1 ± 0.4a	7.1 ± 0.3a	7.0 ± 0.3a	6.7 ± 0.3a+
	Hg alone	-	7.1 ± 0.3a	6.7 ± 0.2a#+	6.6 ± 0.2a+	6.9 ± 0.2a+
	Cd alone	-	6.9 ± 0.3a+	6.8 ± 0.3a+	7.0 ± 0.4a	6.9 ± 0.2a
	Hg + Cd mixture	-	6.3 ± 0.2a+	6.5 ± 0.1a+	7.0 ± 0.3a#	7.2 ± 0.3a

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

The major electrolytes (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were also analysed in the gill, digestive gland, gonad, remaining soft tissue and adductor muscle. There was no observed treatment-dependent effects in any of the tissues (gill, digestive gland, gonad, remaining soft tissue, and adductor muscle) examined, apart for some transient changes within some time point which were lost by the end of the experiment (Tables 19-22). For example, the Na^+ concentration in the gill tissue showed a significant decrease in the Hg alone and the Hg plus Cd mixture treatment compared to the control and Cd alone treatment on day 8, but this effect was lost by the end of the experiment (ANOVA, $p < 0.05$, Table 19).

Table 19: Na^+ concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days continuous exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Gill	Control	1897.3 ± 20.1	1605.0 ± 86.6a	1899.9 ± 95.8a	1810.0 ± 99.9a	1672.8 ± 51.2a
	Hg alone	-	1504.2 ± 95.6a	1994.5 ± 278.6a#	1484.4 ± 44.9b#	1361.9 ± 132.9a+
	Cd alone	-	1695.8 ± 75.0a	2560.8 ± 254.4b#+	1744.5 ± 92.0a#	1620.3 ± 106.0a
	Hg + Cd mixture	-	1523.6 ± 104.5a+	2265.7 ± 67.5b#+	1488.0 ± 57.7b#+	1727.1 ± 208.5a
Digestive gland	Control	778.3 ± 83.9	565.2 ± 81.9a	758.6 ± 118.8a	617.9 ± 18.2a	800.4 ± 108.3a
	Hg alone	-	840.6 ± 188.0a	777.4 ± 132.5a	717.0 ± 66.9a	851.0 ± 97.7a
	Cd alone	-	765.6 ± 82.9a	1059.5 ± 180.4a	728.9 ± 169.0a#	636.6 ± 91.3a
	Hg + Cd mixture	-	870.6 ± 181.2a	844.9 ± 130.0a	664.7 ± 45.1a	891.1 ± 225.3a
Gonad	Control	637.7 ± 149.4	472.9 ± 76.9a	669.7 ± 215.8a	542.9 ± 51.6a	734.2 ± 161.1a
	Hg alone	-	994.4 ± 370.0a	832.0 ± 259.6a	783.4 ± 81.9a	852.3 ± 169.9a
	Cd alone	-	668.8 ± 87.8a	12320 ± 274.3a	635.7 ± 154.8a	720.8 ± 130.3a
	Hg + Cd mixture	-	732.2 ± 178.6a	926.4 ± 235.5a	870.2 ± 133.7a	1163.8 ± 436.1a

Continuation of Table 19: Na^{+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days continuous exposure.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Remaining Soft tissue	Control	832.0 ± 93.9	674.8 ± 76.8a	785.4 ± 94.5a	841.2 ± 28.1a	802.2 ± 99.9a
	Hg alone	-	863.6 ± 78.2a	804.1 ± 93.8a	930.6 ± 119.9a	1004.0 ± 106.9a
	Cd alone	-	924.3 ± 100.7a	965.0 ± 106.4a	787.1 ± 68.8a	859.0 ± 78.6a
	Hg + Cd mixture	-	1044.5 ± 243.5a	962.7 ± 135.2a	914.6 ± 34.8a	905.2 ± 86.9a
Adductor muscle	Control	655.4 ± 65.8	598.1 ± 42.6a	650.6 ± 76.4a	617.8 ± 46.7a	611.0 ± 66.8a
	Hg alone	-	790.9 ± 176.4a	849.1 ± 126a	705.5 ± 100.5ab	667.7 ± 89.8a
	Cd alone	-	562.3 ± 24.0a	952.2 ± 186.5a	811.8 ± 220.5a	566.6 ± 38.5a
	Hg + Cd mixture	-	810.9 ± 208.2a	898.0 ± 153.8a	896.1 ± 90.0b	810.0 ± 152.3a

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

Table 20: K^+ concentrations (µmol g^{-1} dw) in *M. galloprovincialis* after 14 days continuous exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$; 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Gill	Control	406.5 ± 24.9	330. 8 ± 19.0a	437.8 ± 21.8a	334.6 ± 20.2a	361.6 ± 16.8a
	Hg alone	-	340.8 ± 26.9a	441.8 ± 12.9a#	364.5 ± 17.8b	300.6 ± 32.6a#+
	Cd alone	-	331.2 ± 9.3a	527.9 ± 49.8a#	426.3 ± 24.5a	357.6 ± 17.2a
	Hg + Cd mixture	-	318.7 ± 15.6a+	545.7 ± 32.5a#+	370.4 ± 15.8b#	407.3 ± 39.3a
Digestive gland	Control	351.0 ± 26.8	273.9 ± 23.5a	326.6 ± 32.9a	282.8 ± 14.3a	328.4 ± 35.2a
	Hg alone	-	353.3 ± 114.3a	344.5 ± 30.5a	278.2 ± 9.5a	283.1 ± 14.3a
	Cd alone	-	296.9 ± 23.0a	399.9 ± 58.7a	254.8 ± 9.3a#+	254.7 ± 21.6a+
	Hg + Cd mixture	-	265.0 ± 19.7a	347.9 ± 42.2a	286.3 ± 17.3a	276.5 ± 11.4a
Gonad	Control	255.8 ± 33.4	208.6 ± 28.2a	187.0 ± 18.7a	218.3 ± 18.8a	255.7 ± 55.9a
	Hg alone	-	312.6 ± 117.5a	228.9 ± 81.3a	269.9 ± 12.1a	252.1 ± 46.3a
	Cd alone	-	253.1 ± 43.1a	396.1 ± 85.2a	228.8 ± 35.4a	242.0 ± 32.1a
	Hg + Cd mixture	-	248.7 ± 46.5a	313.3 ± 45.6a	305.1 ± 26.3a	239.0 ± 18.4a

Continuation of Table 20: K^+ concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days continuous exposure.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Remaining	Control	242.6 ± 9.1	213.7 ± 13.8a	231.7 ± 13.2a	239.3 ± 4.0a	236.7 ± 14.6a
Soft tissue	Hg alone	-	245.8 ± 44.5a	245.3 ± 15.2a	249.9 ± 6.1a	232.0 ± 17.8a
	Cd alone	-	245.0 ± 11.0a	254.5 ± 19.0a	227.5 ± 8.6a	233.8 ± 11.6a
	Hg + Cd mixture	-	233.6 ± 16.0a	253.4 ± 9.3a	261.6 ± 7.8a	250.1 ± 10.1a
Adductor	Control	322.5 ± 24.8	261.3 ± 8.8a	312.2 ± 33.2a	289.8 ± 9.4a	275.5 ± 13.0ab
muscle	Hg alone	-	284.6 ± 36.8a	371.2 ± 15.6a#	279.1 ± 6.9a#	254.8 ± 2.7b+
	Cd alone	-	261.9 ± 11.0a+	362.8 ± 47.6a	270.1 ± 3.9b+	249.5 ± 10.0b+
	Hg + Cd mixture	-	272.1 ± 15.1a	367.8 ± 25.1a	383.1 ± 44.7b	303.3 ± 13.9a

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

Table 21: Ca^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days continuous exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$; 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Gill	Control	100.6 ± 14.2	69.5 ± 4.1a	83.1 ± 5.1a	84.3 ± 6.1a	76.0 ± 2.4a
	Hg alone	-	68.7 ± 2.1a+	88.9 ± 11.7a#	67.8 ± 2.2b#+	60.2 ± 7.4a+
	Cd alone	-	77.1 ± 3.8a	132.9 ± 13.1b#+	81.9 ± 3.4a#	77.6 ± 6.2a
	Hg + Cd mixture	-	70.1 ± 3.8a+	100.8 ± 2.7a#	69.0 ± 2.8b#+	84.1 ± 12.1a
Digestive gland	Control	61.9 ± 7.9	39.9 ± 4.9a	51.0 ± 7.4a	48.7 ± 5.4a	51.3 ± 7.6a
	Hg alone	-	48.7 ± 6.2a	52.0 ± 7.4a	42.9 ± 3.4a	54.3 ± 6.8a
	Cd alone	-	49.3 ± 2.1a	70.8 ± 13.3a	48.0 ± 10.1a#	37.6 ± 4.2a
	Hg + Cd mixture	-	54.7 ± 8.0a	77.0 ± 2.5 a	42.0 ± 3.6a	46.0 ± 5.4a
Gonad	Control	43.4 ± 7.0	58.6 ± 24.5a	45.1 ± 11.5a	33.6 ± 2.4a	42.0 ± 10.0a
	Hg alone	-	45.2 ± 6.8a	43.1 ± 10.7a	40.3 ± 4.6a	48.9 ± 11.1a
	Cd alone	-	41.1 ± 4.6a	73.9 ± 20.8a	35.7 ± 7.0a	40.2 ± 8.2a
	Hg + Cd mixture	-	132.4 ± 55.9a	51.4 ± 14.0a	48.8 ± 1.1a	40.3 ± 2.6a

Continuation of Table 21: Ca^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days continuous exposure.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Remaining Soft tissue	Control	52.6 ± 6.3	77.9 ± 18.0a	47.2 ± 5.7 a	63. 4 ± 5.5ab	54.4 ± 5.7a
	Hg alone	-	67.7 ± 10.5a	60.5 ± 9.4a	89.9 ± 15.9b	70.8 ± 10.8a
	Cd alone	-	58.7 ± 2.5a	70.5 ± 10.0a	48.6 ± 3.2a	61.2 ± 8.8a
	Hg + Cd mixture	-	90.3 ± 22.8a	84.7 ± 20.1 a	67.7 ± 7.5ab	95.1 ± 18.3a
Adductor muscle	Control	41.5 ± 5.5	41.7 ± 8.3a	43.5 ± 9.1a	38.5 ± 4.8a	37.0 ± 6.2a
	Hg alone	-	43.7 ± 5.6a	48.5 ± 7.4a	43.2 ± 3.8a	44.3 ± 7.4a
	Cd alone	-	37.2 ± 2.9a	56.9 ± 11.7a	46.2 ± 10.7a	31.7 ± 2.8a
	Hg + Cd mixture	-	41.3 ± 2.8a	40.9 ± 3.0a	55.6 ± 7.4a	39.1 ± 2.0a

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

Table 22: Mg^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days continuous exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$; 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Gill	Control	330.4 ± 20.8	283.0 ± 14.8a	346.2 ± 15.9a	302.2 ± 9.1a	300.2 ± .3a
	Hg alone	-	267.5 ± 13.7a+	309.0 ± 24.8a	270.1 ± 7.3b+	246.6 ± 32.0a+
	Cd alone	-	296.1 ± 12.0a	490.2 ± 25.7b#+	314.9 ± 16.0a#	293.1 ± 16.6a
	Hg + Cd mixture	-	270.4 ± 17.5a	414.0 ± 15.0 b#+	270.2 ± 9.4 b#	341.9 ± 37.5a#
Digestive gland	Control	148.3 ± 14.5	110.9 ± 15.4a	146.1 ± 22.1a	119.8 ± 3.4a	153.1 ± 20.4a
	Hg alone	-	156.0 ± 33.4a+	127.8 ± 8.2a	133.9 ± 10.4a	157.0 ± 16.9a
	Cd alone	-	143.4 ± 16.0a	199.6 ± 32.9a	107.7 ± 7.8a	121.7 ± 15.9a
	Hg + Cd mixture	-	156.3 ± 30.5a	162.9 ± 23.6a	125.9 ± 8.6a	131.1 ± 12.3a
Gonad	Control	114.0 ± 24.7	87.8 ± 13.3a	82.7 ± 11.1a	99.1 ± 9.7 a	132.2 ± 26.8a
	Hg alone	-	118.4 ± 14.6a	144.3 ± 42.4a	139.1 ± 13.7a	152.1 ± 32.0a
	Cd alone	-	155.5 ± 39.6a	217.8 ± 47.3a	87.8 ± 8.7 a	129.1 ± 22.2a
	Hg + Cd mixture	-	129.3 ± 31.7a	129.9 ± 19.8a	150.1 ± 21.5a	127.7 ± 14.8a

Continuation of Table 22: Mg^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days continuous exposure.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Remaining Soft tissue	Control	145.2 ± 14.1	123.4 ± 12.3a	142.2 ± 15.7a	151.0 ± 4.1a	148.9 ± 16.9a
	Hg alone	-	152.8 ± 13.7a	145.9 ± 15.7a	166.0 ± 19.3a	176.4 ± 17.8 a
	Cd alone	-	161.9 ± 17.0a	169.9 ± 74a	141.7 ± 11.6a	155.9 ± 12.6a
	Hg + Cd mixture	-	143.5 ± 15.3a	151.2 ± 8.7 a	162.1 ± 6.1a	129.4 ± 5.6a
Adductor muscle	Control	127.0 ± 12.5	113.1 ± 7.5a	126.4 ± 14.9a	122.0 ± 8.6a	118.5 ± 11.4a
	Hg alone	-	147.0 ± 29.1a	160.8 ± 21.1a	131.1 ± 16.0a	125.6 ± 14.6a
	Cd alone	-	121.6 ± 13.6a	178.8 ± 32.9a	114.9 ± 3.2b	110.0 ± 7.1a
	Hg + Cd mixture	-	147.3 ± 31.9a	171.3 ± 25.8a	167.3 ± 17.4c	163.3 ± 13.4a

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

5.2.4. Effects of Hg plus Cd Mixture on TBARS

Tissue (gill, digestive gland, gonad, and adductor muscle) TBARS concentrations were measured at the beginning and the end of the experiment. There was some treatment-dependent statistically significant increases in the TBARS concentration compared to the control (means ± SEM, $n = 6$, ANOVA, $p < 0.05$, Figure 24). The Hg alone treatment showed significant increases in the TBARS concentration in all the tissues examined compared to the other treatments including the controls and the initial stock (ANOVA, $p < 0.05$). For example, TBARS concentration in the Hg alone treatment was significantly increased (> 2 fold) in all tissues examined compared to the Cd alone treatment.

No significant difference was observed in the Cd alone treatment compared to the control in any of the tissues examined (ANOVA, $p > 0.05$, Figure 24). There was a significant decrease in TBARS concentration in the Cd alone compared to the Hg alone, or Hg and Cd mixture treatment, in the gill and digestive gland.

Likewise, the Hg and Cd mixture showed a significant increase in TBARS concentration in the digestive gland and gill, but not the gonad and adductor muscle compared to the control (ANOVA, $p < 0.05$). Exposure to a Hg plus Cd mixture resulted in statistically significant antagonistic interaction in most of the tissues examined (Table 23).

5.2.5. Effects of Hg plus Cd mixture on Total Glutathione Concentration

Total glutathione were measured at the start and end of the experimental period (Figure 25). There was no treatment or time-dependent significant differences in the total glutathione concentration in the gonad and the adductor mussel for all treatments including the control (means ± SEM, $n = 6$, ANOVA, $p > 0.05$, Figure 25). Total

glutathione was totally depleted in the Hg alone treatment in the gill and digestive gland but not the gonad and adductor mussel. In the adductor mussel and gonad, the Hg alone treatment was not significantly different from the control or the other metal treatments (ANOVA, $p > 0.05$).

Like the Hg alone treatment, there was no significant difference in the Cd alone compared to the other treatments including the control (ANOVA, $p > 0.05$) in the gonad and adductor mussel. However, in the gill and digestive gland, total glutathione was significantly decreased and increased respectively, compared to the control (ANOVA, $p < 0.05$).

In the Hg and Cd mixture group, total glutathione concentration was totally depleted in the gill and digestive gland, but not in the gonad and adductor muscle. There was no significantly different in the total glutathione concentration in the gonad and adductor muscle in the Hg plus Cd mixture compared to the other treatments including the control (ANOVA, $p > 0.05$, Figure 25).

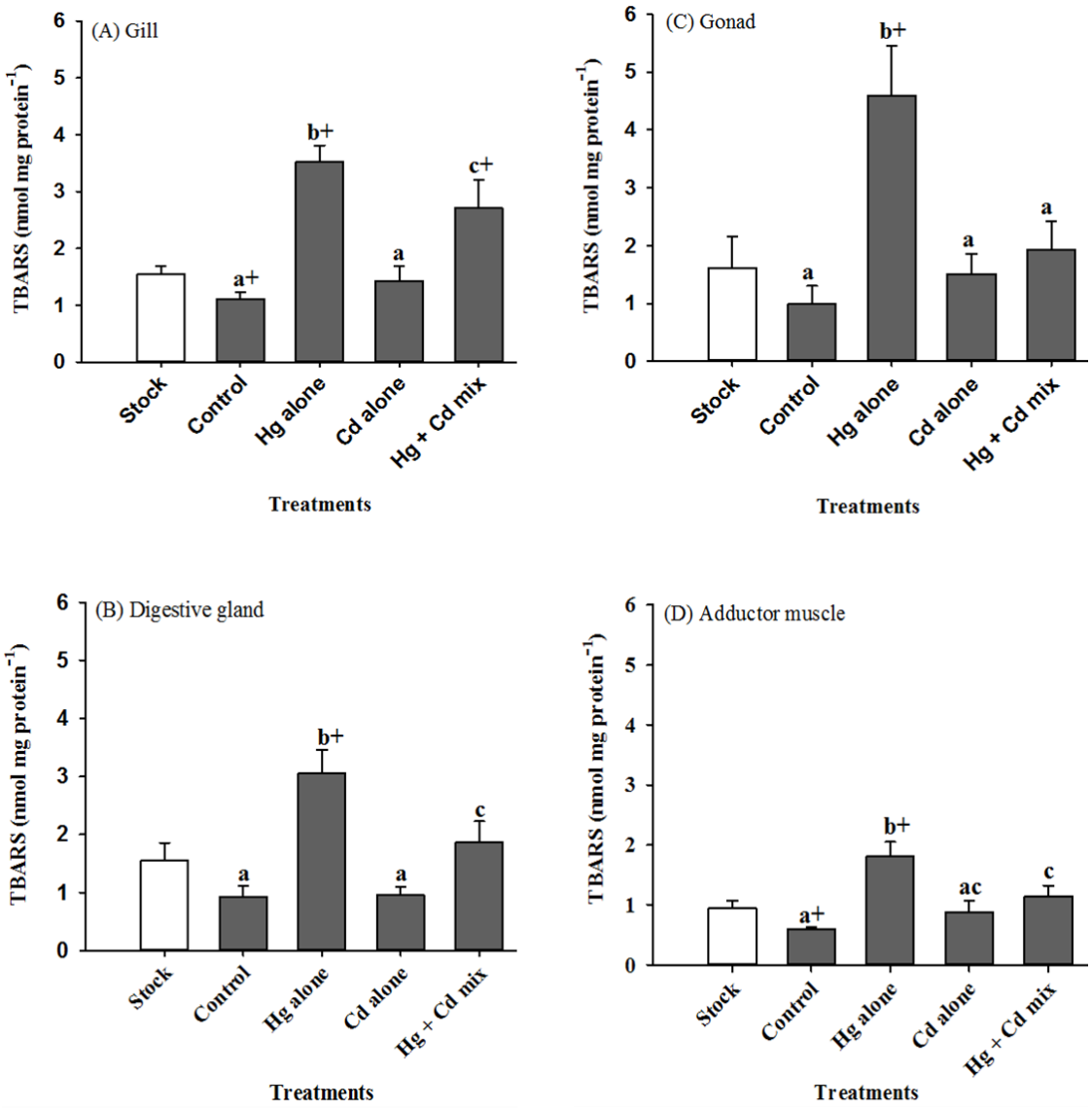


Figure 24: Thiobarbituric acid reactive substances (TBARS) concentration in the supernatant of the (A) gill, (B) digestive gland, (C) gonad, and (D) posterior adductor muscle after 14 days (grey bar) continuous exposure to control (no added metal), 50 µg l^{-1} µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture. The white bar represents the unexposed (initial) mussel at the start of the experiment at time zero. Data are means ± SEM, nmol mg $protein^{-1}$ for n = 4-6 mussel per treatment at each exposure day. Different letters within exposure day indicates statistically significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, stock mussel; ANOVA, $p < 0.05$).

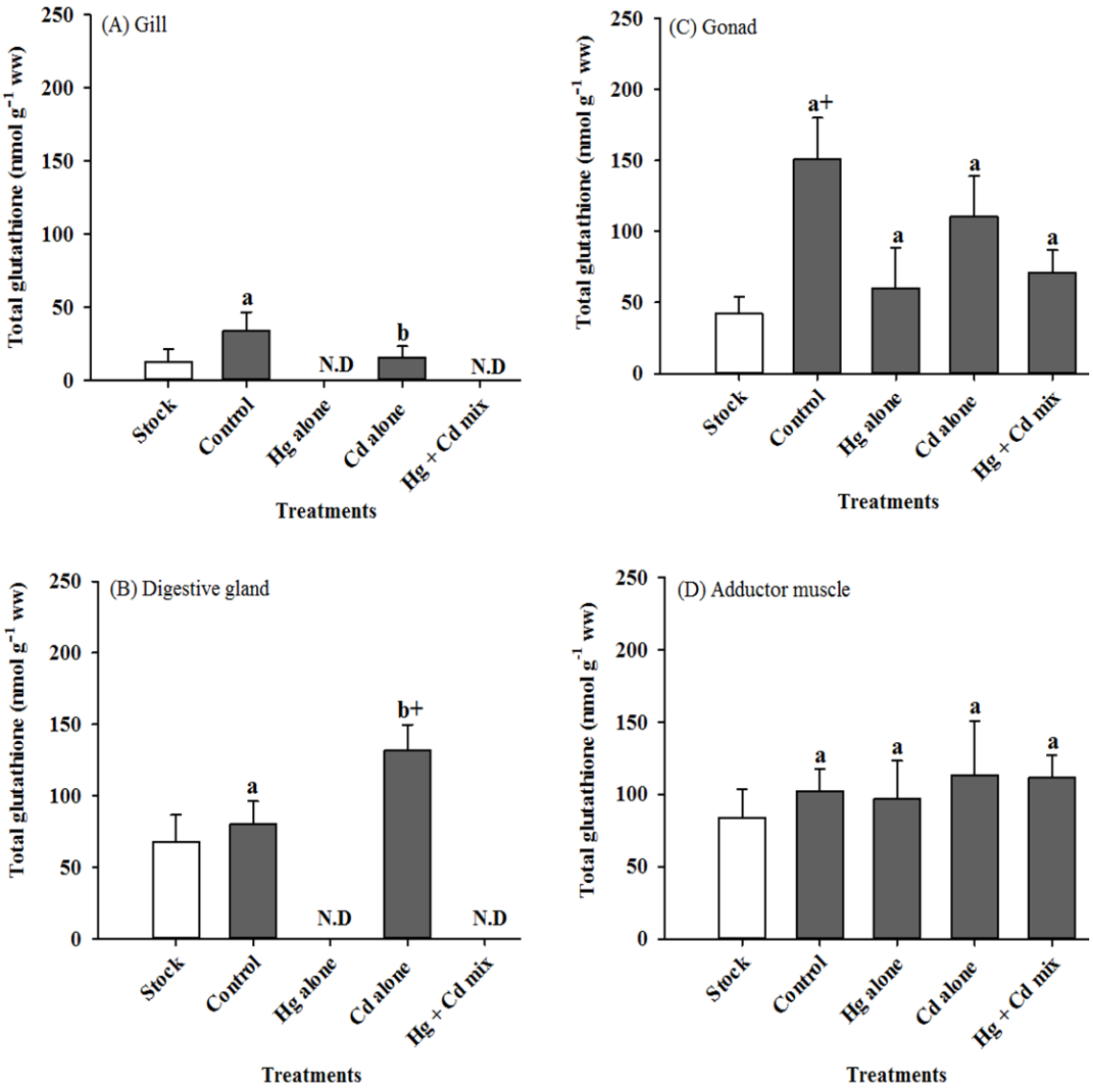


Figure 25: Total glutathione concentration in the supernatant of the (A) gill, (B) digestive gland, (C) gonad, and (D) posterior adductor muscle after 14 days (grey bar) continuous exposure to control (no added metal), 50 µg l^{-1} µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture. The white bar represents the unexposed (initial) mussel at the start of the experiment at time zero. Data are means ± SEM, nmol g^{-1} ww for n = 4-6 mussel per treatment at each exposure day. Different letters within exposure day indicates statistically significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, stock mussel; ANOVA, $p < 0.05$). Note N.D represents not detected.

Table 23: The combined effect of Hg + Cd mixture on TBARS concentration (nmol mg protein^{-1}) in the tissue supernatant of *M. galloprovincialis* at the end of a 14 day continuous exposure to 50 µg l^{-1} Hg alone, 50 µg l^{-1} Cd alone or 50 µg l^{-1} each for the Hg plus Cd mixture.

Tissues	Hg alone (nmol mg protein^{-1})	Cd alone (nmol mg protein^{-1})	Sum of the individual metals	Hg + Cd Mixture (nmol mg protein^{-1})	*p*-value	Mixture Effect
Gill	3.5 ± 0.2	1.4 ± 0.2	4.9 ± 0.4	2.7 ± 0.5	0.002	Antagonistic*
Digestive gland	3.0 ± 0.4	0.9 ± 0.1	3.9 ± 0.5	1.8 ± 0.3	0.08	Additive
Gonad	4.5 ± 0.8	1.5 ± 0.3	6.0 ± 1.1	1.9 ± 0.5	0.002	Antagonistic*
Adductor muscle	1.8 ± 0.2	0.9 ± 0.1	2.7 ± 0.3	1.2 ± 0.1	0.001	Antagonistic*

Data are (mean ± SEM, n = 6/treatment). * represents statistical significance difference (Student's t-test, $p < 0.05$).

5.2.6. Histological Alterations during Continuous Exposure to Hg and Cd Mixture

The histological examination was performed on the gill and digestive gland of *M. galloprovincialis* at the end of a 14 day exposure to Hg and Cd singly or in combination (50 µg l^{-1} each). The gills filament of the control mussels showed normal structure of the frontal face. There was no evidence of hyperplasia, haemocyte infiltration or any other pathology (6/6, Figure 26A). All mussels (6/6) in the Hg alone treatment showed hyperplasia characterised by increased cell in the tips of the lamella and oedema characterised by swelling of the gill lamella (Figure 26B). One out of six mussels showed desquamation of the epithelial cells. Another one out of six mussels showed hypertrophy characterised by increase in some parts of the gill lamella due to enlargement of the epithelial cells in those areas.

Like the Hg alone, the Cd alone treatment also resulted in hyperplasia in all six mussels examined. However, there was no evidence of other damage in the gills as a result of the Cd exposure (Figure 26C). All six mussels in the Hg plus Cd mixture showed hyperplasia as well as oedema of the gill lamella. Like the Hg alone treatment, 1/6 mussel showed desquamation of the gill epithelial cells as well as hypertrophy (6/6, Figure 26D).

Histological examination of the digestive gland of the control mussels showed the normal structure of the digestive tubules (oval or round) (6/6). There was no evidence of haemocyte infiltration, desquamation or any other damage (Figure 26E). In contrast, there were some treatment-dependent pathology in the metal(s) treated groups. The epithelial cell heights of the digestive tubules were statistically significantly increased in the control compared to the metal (s) treatment groups (ANOVA, p = 0.015). However, there was no significant difference between the metal (s) treatment groups (ANOVA, $p > 0.05$). The values were (means ± SEM, n = 6, µm) 37.0 ± 2.6, 28.7 ± 2.5, 23.2 ± 2.9 and 22.5 ± 2.8 for control, Hg alone, Cd alone and Hg plus Cd

mixture respectively. The digestive gland of mussels treated with Hg alone showed haemocyte infiltration in the connective tissues (2/6). Two showed granulocytomas characterised by a dense aggregation of cell (granulocytes and basophilic) in the connective tissue. One of six mussels showed degeneration of the digestive tubules (Figure 26F).

In the Cd alone treatment, 4/6 mussels showed slight haemocyte infiltration in the connective tissue and 1/6 mussels showed desquamation of the digestive cells (Figure 26G). Exposure to mixture of Hg plus Cd resulted histological alterations in the digestive gland of *M. galloprovincialis*. Four out of 6 mussels showed granulocytomes, 2/6 showed slight inflammation characterised by haemocyte infiltration and 1/6 showed desquamation of the digestive cells. The binary combination of Hg plus Cd resulted in an antagonistic interaction in epithelial cell heights of the digestive tubules.

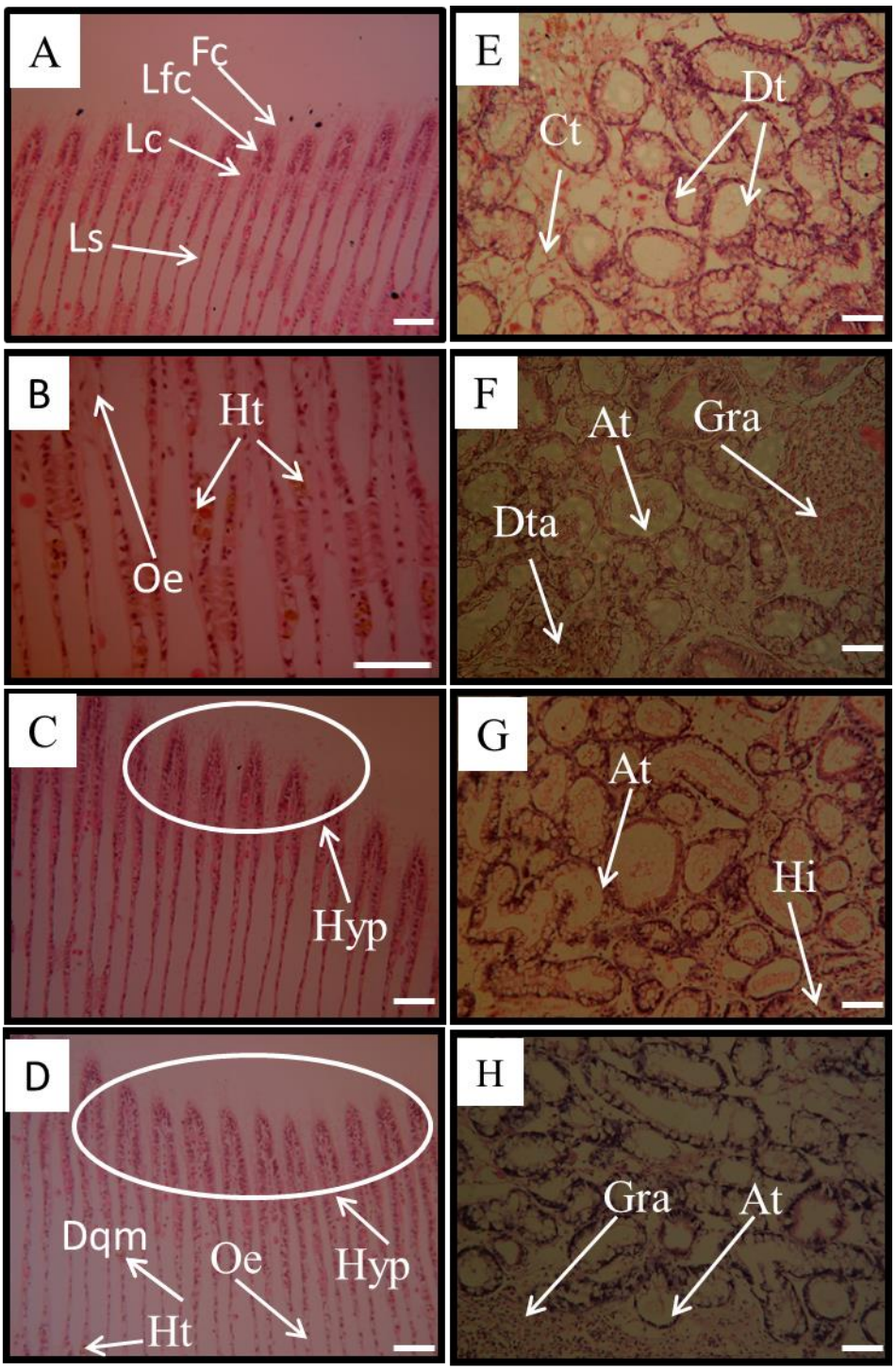


Figure 26: Histology of the gill (A-D) and digestive gland (E-H) of *M. galloprovincialis* after 14 days continuous exposure to control (no added metal, A, E), 50 µg l^{-1} Hg as $HgCl_2$ (B, F), 50 µg l^{-1} Cd as $CdCl_2$ (C, G) or 50 µg l^{-1} Hg plus Cd mixture (D, H). Slides were stained with Mayer's Haemotoxylin and eosin. Circle represents gill filaments with hyperplasia (Hyp) on the tips. At = atrophy; Ct = connective tissues; Dt = digestive tubules; Dqm = desquamation; Dta = degeneration of tubule architecture; Ff = frontal face; Fc = frontal cilia; Lfc = laterofrontal cilia; Lc = lateral cilia; Ls = lacuna space; Hyp = Hyperplasia; Ht = Hypertrophy; Oe= oedema. Scale bar: 50 µm.

5.3. Discussion

This study provides the first report on the combined effect of Hg and Cd on tissue accumulation and sub-lethal physiological effects on marine mussels, *M.galloprovincialis* exposed to equal peak concentrations of Hg and Cd singly or in combination for up to 14 days. Overall, the results have demonstrated that *M.galloprovincialis* can accumulate higher levels of Hg and/or Cd in an aqueous exposure to metals singly or in combination compared to the controls (Figures 21-22). Target organs for Hg or Cd are the same whether the metal is exposed singly or in combination with the highest in the gill and digestive gland and the adductor muscle showing the least.

5.3.1. Water Quality During Aqueous Exposure to a Mixture of Hg and Cd

Daily water quality measurements were not significantly different in all treatments including the control tanks. The nominal concentration of 50 µg l^{-1} per metal in the seawater was confirmed by the measured concentrations (Figure 20). The targeted metal concentrations in the seawater were achieved and were not significantly different from each other. Values (means ± SEM) were 47.3 ± 1.8, 54.8 ± 2.1, 48.0 ± 1.3, 51.8 ± 0.9 µg l^{-1} for Hg alone, Cd alone, Hg (Hg plus Cd) and Cd (Hg plus Cd) mixture respectively. Together these suggest that the seawater quality in the tanks were similar and there were no stress related effects based on the parameters measured.

5.3.2. Tissue Accumulation and Metals Interaction during Exposure to a Mixture of Hg plus Cd

The results of the present study, showed no treatment-dependent significant differences in the accumulation of the individual metals compared to the mixtures in all tissues examined except for the Cd accumulation in the gill (Figures 21 and 22). The gill showed a significant increase in Cd accumulation in the Hg plus Cd mixture compared to the Cd alone treatment. This significant increase of Cd in the gill of the Hg plus Cd mixture could probably suggest that the presence of Hg seems to positively influence the accumulation of Cd in the gill tissue. The total metal concentration in the mixture showed an increasing trend of accumulation (Figure 23) that were not significant, indicating additive (non-interaction) effect. The additive effect suggests that Hg and Cd were taken up through independent pathways. However, the accumulation of Hg was much higher compared to Cd in all the tissues examined. For example in the gill, Hg accumulation in the Hg plus Cd mixture after 14 days was more (approximately 11 fold) compared to the Cd of the same mixture. In mussels, dissolved metal uptake mainly occurs through the gills, in the mucus-rich abfrontal (distal third of the gill filament) and laterofrontal regions of the gill filaments (Marigómez et al., 2002; Varotto et al., 2013). Cd enters the gill epithelial cells by passive diffusion (Carpene and George, 1981) or facilitated diffusion through the Ca^{2+} channels (oyster, Roesijadi and Unger, 1993) with the inward concentration gradient maintained by intracellular binding and sequestration of Cd ions, (George, 1981). Pathways for Hg uptake across the gills includes Na^{+} (Holye and Handy, 2005), Na^{+} and/or Ca^{2+} (Busselberg, 1995) channels, and methylation of Hg from inorganic to methylmercury for uptake by diffusion of the organic form of mercury. The high tissue Hg accumulation observed in the present study (Figure 21) may be due to its potency to stimulate both the proliferation of mucus cells on the gill and the secretion of mucus into the water. The binding of inorganic Hg

to the mucus, should protect the epithelium and reduce the bioavailability of the metal (Part and Lock 1983). However, mucus might also precipitate, leading to an apparent increase of metal in/on the gill tissue.

Similar reports of increased Hg concentrations in the tissues have been made with Hg co-exposed with Cu for 11 day during additional 4 and 7 days depuration *M. galloprovinciallis* (Raftopoulou and Dimitriadis, 2011). They suggested that the independent accumulation of the Hg and Cu might be as a result of the dissimilar ability Hg or Cu to induce metallothioniens, or on the ability of Hg to bind cell ligands such as glutathione in the gills and digestive gland.

Also, Hg binds to -SH group more readily than Cd with a covalent index of 4.08 and 2.71 for Hg and Cd respectively (van Kolck et al., 1996). In the present study, MT was not measured but apparent depletion of total glutathione in the gills might be as a result of complexes with Hg. The idea is consistent with a previous suggestion made by Raftopoulou and Dimitriadis (2011) on Hg binding to ligands such as GSH. Inorganic mercury forms a stable 1:2 complex with glutathione at a stability constant of log K = 40.96 (Stricks and Kolthoff 1953; see also sections 4.2.5 and 5.3.4) where exposure to Hg resulted in apparent depletion of the total glutathione especially in the gills and digestive gland of *M. galloprovincialis*.

5.3.3. ***Effects of Hg plus Cd Mixture on the Haemolymph Chemistry and Ion Regulation***

The effects of Hg or Cd on the haemocytes of blue mussels have been reported previously (e.g., Sheir et al, 2010; Sheir and Handy 2010). However, the effect of Hg and Cd in mixtures is lacking. In the present study, exposing mussels to Hg or Cd singly or in combination did not result in any significant effect on the total haemocyte

counts, haemolymph protein, cell-free haemolymph glucose. Neither was there any effect on osmotic pressure, Na^+ and K^+ concentrations in the cell-free haemolymph. Also, the major electrolytes (Na^+, K^+, Ca^{2+} and Mg^{2+}) examined in the tissues (gill, digestive gland, remaining soft tissue, gonad and adductor muscle) did not show any difference between the treatments. Together, the result suggests the osmoregulatory ability of the mussels was not altered. Values for electrolytes in the cell-free haemolymph were within the normal range for mussels (Potts, 1954). The values were also consistent with previous reports (Sheir et al., 2010; Sheir and Handy, 2010; Amachree et al., 2013). Also, the mussels were held in normal seawater and not stressed as indicated by the cell-free haemolymph glucose and haemolymph protein concentration. Mussels were kept in normal seawater and thus were not osmotically challenged and were not stressed.

5.3.4. Oxidative Stress and Organ Pathology during Exposure to a Mixture of Hg and Cd

Toxic metals such as Hg and Cd has been shown to induce reactive oxygen species, ROS, which could overwhelm the antioxidant defence mechanisms thus leading to increased lipid peroxidation and subsequent oxidative damage of tissues (Garron et al., 2005; Valavanidis et al., 2006; Tarasub et al., 2011). Lipid peroxidation is considered to be one of the key events in oxidative stress (Botsoglou et al., 1994). In the present study, TBARS concentration at the end of the experiment in the Cd alone treatment was not significantly different from the controls (Figure 24). In contrast, the TBARS concentrations in the Hg alone treatment was significantly increased in all tissues. Likewise, increased TBARS concentration was also seen in the gill and digestive gland of the Hg plus Cd mixture treatment. These increased in the

concentration of TBARS in the Hg alone as well as the Hg plus Cd treatments (Figure 24) suggest Hg-induced oxidative stress in the mussel.

There was no metal induced effect on total glutathione in the gonad and adductor muscle for all treatment including control. Total glutathione was significantly decreased (gill) and increased (digestive gland) in the Cd alone treatment compared to the control, suggesting the total glutathione was stimulated by the exposure. However, despite these changes in total glutathione concentration, there were no associated increases in the TBARS concentration in the same tissue compared to the control, suggesting there was no overt oxidative stress from the Cd exposure. This is not surprising, previous work in our laboratory has confirmed that the concentration and duration of the experiment is not enough to cause increases in TBARS or depletion of total glutathione and resultant oxidative damage (Sheir and Handy, 2010; Amachree et al., 2013).

In the present study, the Hg alone, and Hg plus Cd mixture treatment showed apparent depletion of the total glutathione concentration (Figure 25) as well as increased TBARS concentration in the gill and digestive gland as seen in the histology (Figure 26). Together these suggest oxidative stress and resultant oxidative injury in the gill and digestive gland. The injury in the Hg plus Cd treatment was more severe (6/6 hypertrophy) compared to the Hg alone (1/6 hypertrophy, Figure 26) especially in the gills. There appeared to be no reports of the combined effect of Hg plus Cd on TBARS concentration in mussels. However, epithelial cell damage is believed to be as a result of increased lipid peroxidation (Gstraunthaler et al., 1983; Lund et al., 1993) and both Hg and Cd are known to inducers of oxidative stress. In the present study, hyperplasia was a common irritant due to metal exposure and is consistent with previous report by Coles et al. (1995).

5.3.5. Conclusions

This study provides the first report on the accumulation and sub-lethal effect to *M.edulis* after aqueous exposure to Hg and Cd mixture. Target organs were the same for both Hg and Cd. The result has demonstrated that Hg and Cd when co-exposed did not show interaction in tissue metal accumulation. Though, the sub-lethal bioassays measured in the present thesis were not affected during the mixture exposure, antagonistic (less-than-additive) interaction was observed in the TBARS.

Chapter 6 ***:***

Accumulation and Sub-lethal Physiological Effects on the Blue Mussel, Mytilus galloprovincialis, during Intermittent Exposure to Mercury and Cadmium Mixture

Abstract

In real ecosystem, aquatic organisms are exposed intermittently to a complex cocktail of contaminants. Current hazard assessment practices for chemical mixtures used continuous exposure. The effect of chemical mixtures during intermittent exposure profile is largely unknown. Mussels were exposed intermittently (2 days exposure; 2 days in clean seawater) using a semi-static and triplicated design to either control (no added metal), 50 µg l^{-1} (Hg alone), 50 µg l^{-1} (Cd alone), as well as to a combination of 50 µg l^{-1} Hg plus 50 µg l^{-1} Cd for 14 days. Tissues (gill, digestive gland, gonad, remaining soft tissue, adductor muscles and haemolymph) were collected on days 0, 2, 4, 8 and 14 for metal analysis and sub-lethal responses using a suite of assays. At the end of the experiment, Hg concentration in the haemolymph was significantly higher in the Hg alone compared to the Hg plus Cd treatment. Cd concentration in the gill was lower in the Cd alone compared to the Hg plus Cd mixture. Exposure to a mixture of Hg plus Cd resulted in an additive effect on tissue metal accumulation. For effects, exposure to the metals singly or in mixture did not cause oxidative stress as measured by TBARS and total glutathione in tissue supernatants. Histological observations of the internal organs were similar across the treatments, apart from the Cd alone treatment that showed less inflammation in the digestive gland compared to Hg alone or Hg plus Cd mixture. There were no effects on total haemocyte count, glucose, osmotic pressure and ion concentrations in the tissue and cell-free haemolymph. Haemolymph protein was significantly increased in the Hg alone treatment only. Overall, an additive effect was observed in the metal accumulation and the sub-lethal endpoints measured were not obviously affected. Thus, in terms of risk assessment, the regulation for the individual metals will protect for mixtures of Hg plus Cd in seawater.

6.0. Introduction

In real ecosystem, aquatic organisms are exposed intermittently to a complex cocktail of contaminants. Current hazard assessment practices for chemical mixtures uses data observed from computer simulated mathematical models or laboratory based experimental data assuming a continuous exposure profile, which do not cover for intermittent exposure. As the importance of intermittent exposure is emphasised in risk assessement, mathematical models in particular the toxicokinetic-toxicodynamic (TK-TD) models have been proposed (Ashaeur and Brown, 2013). Although these models simulates sub-lethal effects during intermittent exposure, there is still the debate on which of the model are most suitable. Reports on combined effect of metals on mussels during continuous exposure includes: synergistic effect on the survival, filtration rate, and oxygen consumption of *Perna viridis* (Hg plus Cd, Mohan et al., 1986); antagonistic effect of Cu on Hg accumulation in the digestive gland of *M. galloprovincialis* (Hg plus Cu, Raftopoulou and Dimitriadis, 2011) and antagonistic effects on embryogenesis of *M. galloprovincialis* (Hg plus Cd plus Cu, Prato and Biandolino, 2007). Reports on the sub-lethal effect of the mixture during intermittent exposures are lacking. To the best of our knowledge this is the first report on the sub-lethal effect of mixture toxicity of Hg and Cd during intermittent exposure to mussels.

The study was designed exactly as those of the previous chapter (chapter 5) but for an intermittent (two days exposure; two days placed in clean water). The idea was to assess the interactive toxicity of Hg plus Cd during intermittent using the observed toxicity of the individual metals as a control in the same experiment. The hypothesis is that the arithmetic sum total of the individual metals will be equal to the sum total of the combinations, thus producing an additive effect. The method of assessing the interactive toxicity of the binary metal combination (Hg plus Cd) involved testing the single metals as well as the combination using an equal peak concentration of each

metal. To assess the combined effects, tissue metal accumulation and a range of sub-lethal endpoints relating to the main physiological processes were measured. These included osmoregulation (tissue electrolytes, cell-free haemolymph electrolytes and osmotic pressure), oxidative stress parameters (lysosomal membrane damage via neutral red retention, total glutathione and thiobarbituric acid reactive substances, TBARS), and general animal health (haematology and organ pathology).

6.1. Methodology

6.1.1. Test organisms and acclimation

Mussels with shell length 40-60 mm were collected in October, 2012 from Port Quin and acclimated for two weeks in the Plymouth laboratory as described (Sections 2.1 and 2.2). Water Quality parameters (e.g., pH, salinity, dissolved oxygen and total ammonia) were measured daily in the stock filtered seawater. Stock seawater was changed twice per week during the acclimation period.

6. 1.2. Experimental design

The experimental design was exactly the same as described earlier Chapter 5, section 5.1.2 but with difference in the exposure profile. Metal exposure in the present chapter was performed intermittently (2 day exposure: 2 days placed in clean seawater). Briefly, mussels (means ± SEM, n =132; whole weight, 18.7 ± 0.3 g; length, 52 ± 0.1mm) were randomly selected from the stock and allocated to twelve (12) glass aquaria containing 10 l of filtered seawater.

The quality (pH, salinity, dissolved oxygen, total ammonia) of the test media (seawater) was analysed daily. Samples of the test media were also collected daily

immediately after and before renewal of test media for Hg and/or Cd concentration. The background concentrations in the control filtered seawater (means, n = 42, Figure 6.1) were below the detection limit of the instrument (0.19 ± 0.1 Hg and 0.23 ± 0.1 µg l^{-1} Cd) Mussels (2/test vessel; 6/treatment) were randomly collected at day 2, 4, 8 and 14 for tissue trace elements analysis, plasma ions, osmotic pressure. Addition 6 mussels/treatment were collected at the end of the experiment (day 14) for total haemocyte counts, plasma glucose, tissue total glutathione, tissue TBARS concentration and histological examinations.

6.1.3. Haemolymph extraction and tissue collection

Mussels were collected from glass aquaria on sampling days (2, 4, 8 and 14) rinsed to removed excess Hg and/or Cd. Haemolymph and tissues (adductor muscle, digestive gland, gill, gonad, and remaining soft tissue) were collected and prepared as described (Section 2.4).

6.1.4. Trace metal analysis

The tissues and seawater were analysed for Hg and Cd concentration and electrolyte composition as described (Section 2.5). The procedural detection limit of the instrument for seawater (ICP-MS, means ± SEM, n = 6) were 0.19 ± 0.1 Hg and 0.23 ± 0.1 µg l^{-1} Cd. While those of the tissue digest on the ICP-OES were 7.6 µg l^{-1} Hg and 5.5 µg l^{-1} Cd and was derived from three times the standard deviation of the procedural blank. For a typical 0.1 g of tissue the detection limit equates to 0.38 µg g^{-1} dry weight and 0.29 µg g^{-1} dry weight for Hg and Cd respectively. Calibrations for ICP-OES and

ICP-MS were performed with matrix-matched analytical grade standards containing internal reference materials indium and iridium as described (Section 5.1.4).

6.1.5. Total glutathione and TBARS analysis

Tissue homogenisation, total glutathione and TBARS concentrations were determined exactly as described (Section 5.15).

6.1.6 Total haemocyte count

Total haemocyte of the haemolymph was counted as described (Section 2.10).

6.1.7. Determination of osmotic pressure, plasma ions and glucose concentration

The osmotic pressure, plasma ions and glucose concentration in the cell-free haemolymph were determined exactly as described (Section 2.11 and 2.12).

6.1.8. Calculation and statistical analysis

All statistical analyses were performed by the StatGraphic Plus for windows version 5.1 as described (Section 5.1.8).

6.2. Results

6.2.1. Aqueous Exposure to a Mixture of Hg and Cd

No mortality was observed in mussels exposed intermittently to Hg and Cd singly or in combination. The background concentrations of Hg and Cd in the control filtered water (means, n = 42, µg l^{-1}, Figure 27) were below the detection limit of the instrument (0.23 ± 0.1 0.19 ± 0.1 and for Hg and Cd respectively). The nominal concentration 50 µg l^{-1} of metals (Hg or Cd) was confirmed with the measured concentration from the tanks (Figure 27). Metal concentrations in the water were within target and values (n = 21, µg l^{-1}, means ± SEM) were 50.8 ± 1.5; 52.7 ± 1.8; 50.3 ± 1.7 and 53.5 ± 0.6 for Hg alone, Cd alone, Hg (Hg plus Cd) and Cd (Hg plus Cd) mixture respectively. Cd concentration in the Cd alone treatment were not significantly different from those of the Hg plus Cd mixture (Student's t-test, $p > 0.05$). Neither was the Hg concentration in the Hg alone significantly different from those of the Hg and Cd mixture (Student's t-test, $p > 0.05$).

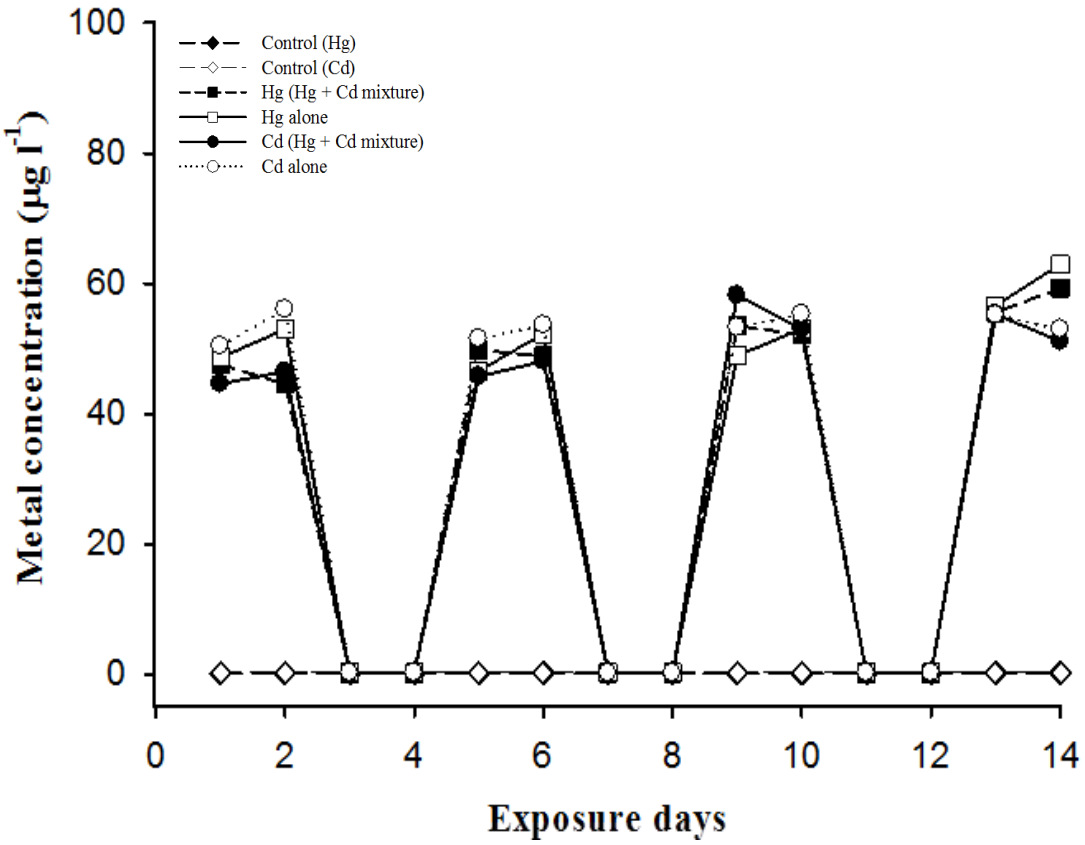


Figure 27: Mercury and cadmium concentration in seawater after 14 days intermittent exposure to control [no added Hg (closed diamond) or Cd (open diamond) on dashed line], 50 µg l^{-1} Hg alone (open squares on solid line), 50 µg l^{-1} Cd alone (open circle on dotted line), or 50 µg l^{-1} Hg (closed squares on dashed line) plus 50 µg l^{-1} Cd (closed circle on solid line) mixture. Water samples were collected immediately after the daily renewal of the test media. Data are means, µg l^{-1} for n = 3 tanks per treatment at each exposure day. Error bars were not shown for clarity.

6.2.2: Tissue Metals Accumulation

All mussels exposed to 50 μg l^{-1} Hg or Cd singly or in combination resulted in a statistically significant tissue (gill, digestive gland, remaining soft tissue, gonad, adductor muscle, haemolymph) metal(s) accumulation compared to the unexposed control, or stock animals (ANOVA, $p < 0.05$).

There was no statistically significant difference between the Hg alone and the Hg plus Cd mixture for Hg accumulation in all tissues apart from the haemolymph by the end of the experiment (ANOVA, $p > 0.05$, Figure 28). The haemolymph showed a significant decrease in Hg accumulation in the Hg plus Cd mixture compared to the Hg alone treatment at the end of the experiment (ANOVA, $p < 0.001$). There were some transient time-dependent increase (digestive gland, day 4) and decrease (adductor muscle, day 8) in the Hg accumulation in the Hg alone compared to the Hg plus Cd mixture (ANOVA, $p < 0.05$, Figure 28), which was lost by the end of the experiment.

Similarly, Cd accumulation was significantly increased in the Cd alone or Hg plus Cd mixture compared to the control or stock mussel for all the tissue examined (ANOVA, $p < 0.001$, Figure 29). There was no significant difference between the Cd alone and the Hg plus Cd mixture for Cd accumulation in all tissues examined apart from the gill by the end of the experiment. Cd accumulation was significantly increased in the Hg plus Cd mixture compared to the Cd alone at the end of the experiment (ANOVA, $p < 0.05$, Figure 29).

The binary exposure did not show any significant mixture interaction (additive effect) on metal accumulation in all tissues examined (Student's t-test, $p > 0.05$, Figure 30).

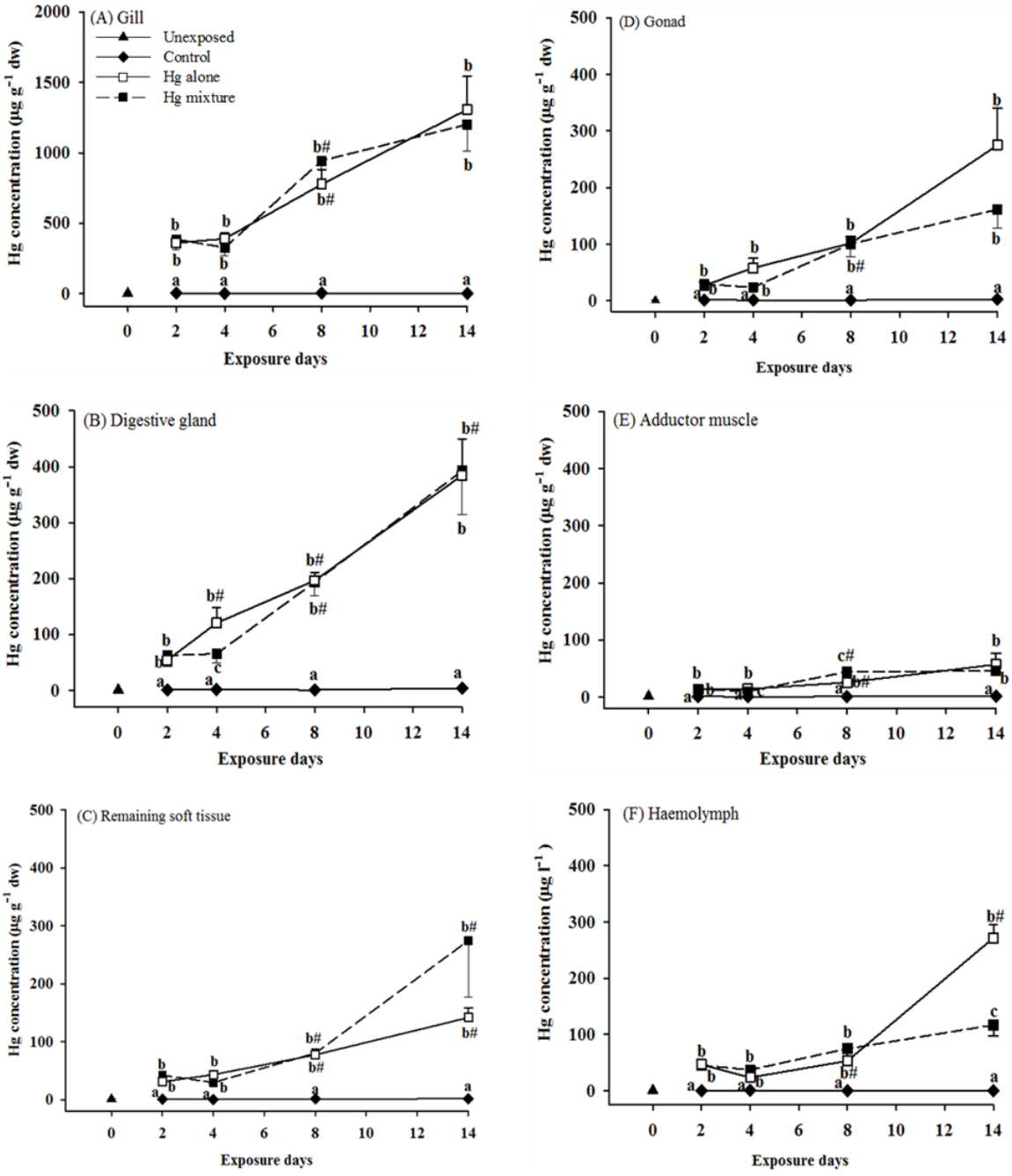


Figure 28: Mercury concentrations in the (A) gill, (B) digestive gland, (C) remaining soft tissue, (D) gonad, (E) posterior adductor muscle and (F) heamolymph after 14 days intermittent exposure to control (no added Hg, diamonds on solid line), 50 µg l^{-1} Hg alone (open squares on solid lines) or 50 µg l^{-1} each for Hg plus Cd mixture (closed squares on dashed lines). The black triangle at time zero represents the background Hg concentration in the unexposed (initial) mussels at the start of the experiment. Data are means ± SEM, µg Hg g^{-1} dry weight tissue, n = 4-6 mussel per treatment at each exposure day. Different letters within the exposure day indicates a significant treatment effect (ANOVA, $p < 0.05$). # indicates a significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, initial mussel stock) (ANOVA, $p < 0.05$).

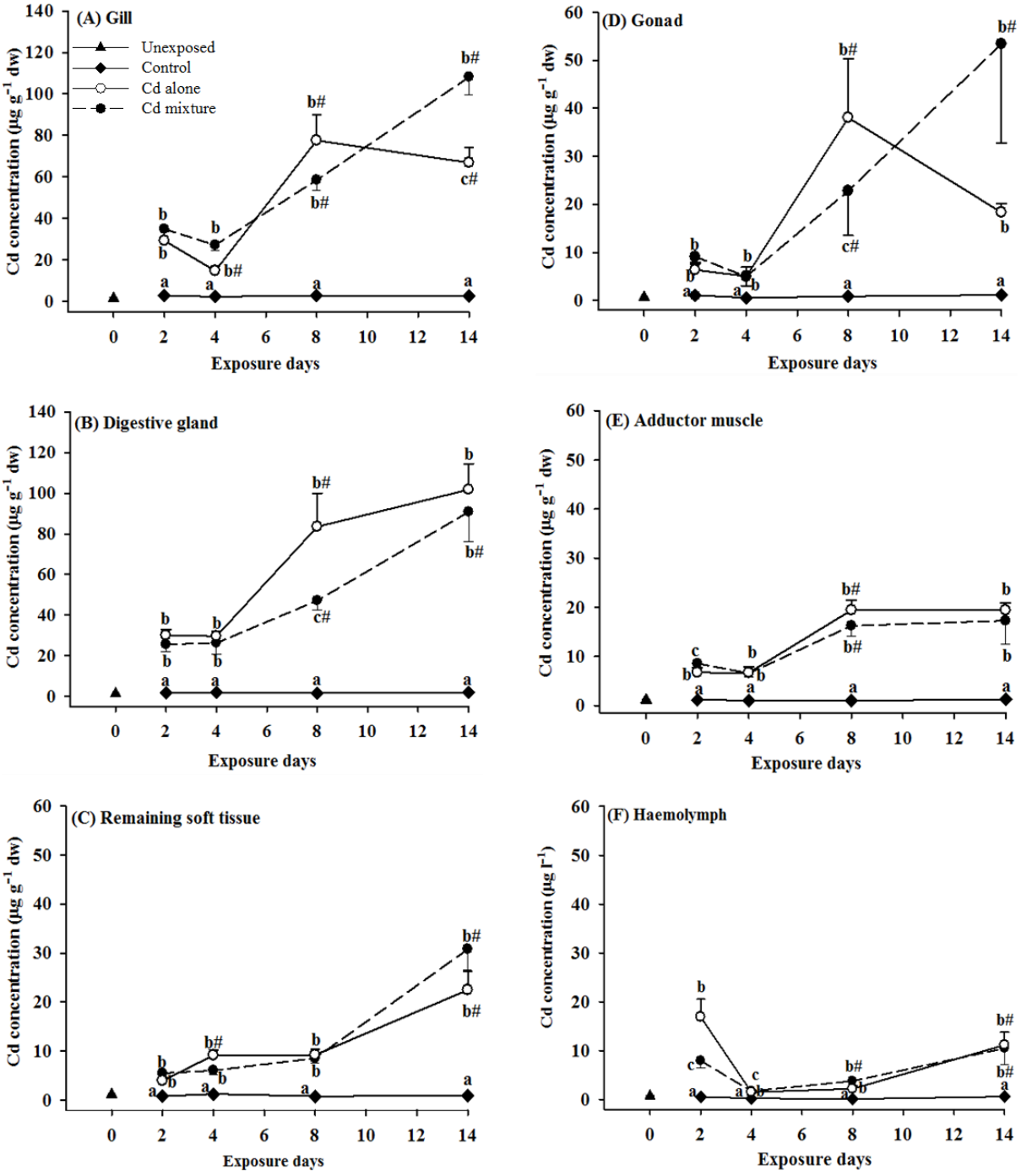


Figure 29: Cadmium concentrations in the (A) gill, (B) digestive gland, (C) remaining soft tissue, (D) gonad, (E) posterior adductor muscle and (F) heamolymph after 14 days intermittent exposure to control (no added Cd, diamonds on solid line), 50 µg l^{-1} Cd alone (open cycles on solid lines) or 50 µg l^{-1} each for Hg plus Cd mixture (closed cycles on dashed lines). The black triangle at time zero represents the background Hg concentration in the unexposed (initial) mussels at the start of the experiment. Data are means ± SEM, µg Cd g^{-1} dry weight tissue, n = 4-6 mussel per treatment at each exposure day. Different letters within the exposure day indicates a significant treatment effect (ANOVA, $p < 0.05$). # indicates a significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, initial mussel stock) (ANOVA, $p < 0.05$).

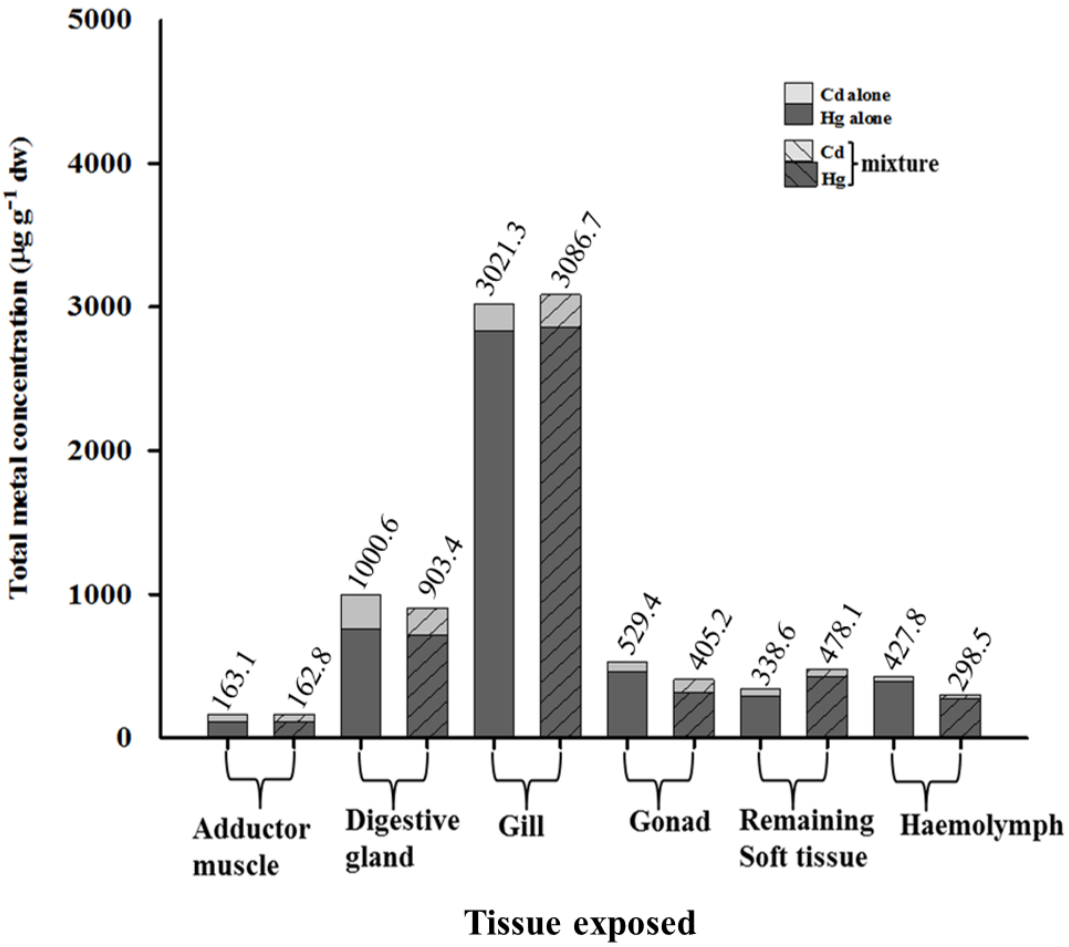


Figure 30: Tissue total metal concentration after 14 days intermittent exposure to individual (Hg or Cd) or mixture (Hg plus Cd mixture). The bars without pattern fill represent the individual metals (Hg, grey and Cd, white). The bars with diagonal pattern fill represent a combination of metals (Hg, grey and Cd, white). The values on each bar represent the arithmetic sum of the metals accumulated in each of the tissue examined.

6.2.3. Effects of Hg plus Cd Mixture on the Haemolymph Chemistry and Ion Regulation

There was no statistically significant difference in the total haemocyte count measured at the beginning and end of the experiment (ANOVA, $p > 0.05$, Table 24). However, the haemolymph protein was significantly increased in the Hg alone treatment (ANOVA, $p < 0.05$, Table 24). There was no significant different in the plasma glucose for all treatment including the controls (ANOVA, $p > 0.05$, Table 24).

There were no treatment-dependent difference between the Hg alone, Cd alone or Hg plus Cd mixture for osmotic pressure, Na^+ or K^+ concentrations in the cell-free haemolymph (ANOVA, $p > 0.05$, Table 25) at the end of the experiment. Overall values were within the normal ranges and are consistent with those previously reported within this thesis for *M. galloprovincialis.*

Tissue Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations were also determined in the gill, digestive gland, gonad, remaining soft tissue and adductor muscle at various exposure times (Table 26-29). All the tissues examined for Na^+ concentration did not show any significant treatment effect by the end of the experiment (ANOVA, $p > 0.05$) apart from the gill. By the end of the experiment, Na^+ concentration in the gill was significantly increased in the Cd alone treatment compared to the other treatments (ANOVA, $p < 0.05$). For the tissue K^+ concentration, only the adductor muscle in all the tissues examined showed a significant treatment-dependent increase (ANOVA, $p < 0.05$, Table 27). Likewise, for Ca^{2+} concentrations only the remaining soft tissue and adductor muscle showed a statistically significant increase in the Ca^{2+} concentration in the Hg alone and Hg plus Cd mixture treatments (ANOVA, $p < 0.05$, Table 6.5). There was no significant difference in Mg^{2+} concentration in all treatments for all tissues examined (ANOVA, $p > 0.05$, Table 29).

Table 24: Total haemocyte counts (THC), whole haemolymph protein and cell-free (plasma) glucose concentration in haemolymph from *M.galloprovincialis* after 14 days intermittent exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$, and 50µg l^{-1} each for Hg plus Cd mixture.

Parameters	Treatments	Exposure days	
		0	14
THC (*10^6 cells ml^{-1})	No metal control	5.11 ± 1.08	3.54 ± 1.14a
	Hg alone	-	2.40 ± 0.56a
	Cd alone	-	3.37 ± 0.99a
	Hg + Cd mixture	-	2.57 ± 0.46a
Haemolymph Protein (mg ml^{-1})	No metal control	0.30 ± 0.07	0.45 ± 0.13a
	Hg alone	-	0.79 ± 0.13b+
	Cd alone	-	0.33 ± 0.06a
	Hg + Cd mixture	-	0.38 ± 0.07a
Plasma Glucose (mmol l^{-1})	No metal control	0.29 ± 0.04	0.24 ± 0.05a
	Hg alone	-	0.38 ± 0.06a
	Cd alone	-	0.35 ± 0.05a
	Hg + Cd mixture	-	0.32 ± 0.03a

Data are means ± SEM, n = 6 mussels per treatment per exposure day. Different letters within each exposure day indicates significant treatment effect (ANOVA, $p < 0.05$). + indicates significant time effect compared to day 0 (stock mussels, ANOVA, $p < 0.05$).

Table 25: Osmotic pressure, Na^+ and K^+ concentration in the cell-free haemolymph from *M.galloprovincialis* after 14 days intermittent exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$; 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for Hg plus Cd mixture.

Parameters	Treatments	Exposure days				
		0	2	4	8	14
Osmotic Pressure (mosmol kg^{-1})	No metal control	1028.8 ± 6.7	1006. 8 ± 6.2a	897.0 ± 23.5a#+	948.5 ± 4.7a#+	920.2 ± 24.4a+
	Hg alone	-	1014.6 ± 4.0a	870.3 ± 1.8a#+	955.4 ± 4.9a#+	952.8 ± 10.1a+
	Cd alone	-	993.5 ± 9.4a+	883.0 ± 6.0a#+	955.7 ± 6.7a#+	956.5 ± 6.2a+
	Hg + Cd mixture	-	1015.7 ± 8.1a+	882.3 ± 9.1a#+	963.8 ± 4.8a#+	948.8 ± 3.0a+
Na^+ (mmol l^{-1})	No metal control	436.9 ± 6.6	420.0 ± 4.1a	455.9 ± 4.2a	499.0 ± 4.7a	458.0 ± 9.8a
	Hg alone	-	431. 3 ± 10.4a	461.1 ± 5.5a	498.4 ± 4.0a	465.8 ± 2.2a
	Cd alone	-	434.2 ± 8.1a	454.4 ± 5.0a	475.5 ± 14.4a	451.9 ± 15.4a
	Hg + Cd mixture	-	429.9 ± 5.1a	468.7 ± 4.1a	464.7 ± 11.3a	446.6 ± 14.4a
K^+ (mmol l^{-1})	No metal control	8.3 ± 0.2	8.2 ± 0.1a	7.1 ± 0.3a#+	5.8 ± 0.3a#+	6.4 ± 0.4a+
	Hg alone	-	8.2 ± 0.3a	6.4 ± 0.2a#+	6.8 ± 0.1b#+	7.0 ± 0.1a+
	Cd alone	-	8.7 ± 0.3a	6.5 ± 0.1a#+	7.5 ± 0.2b#	7.2 ± 0.3a+
	Hg + Cd mixture	-	8.1 ± 0.1a	5.4 ± 0.1b#+	7.8 ± 0.4b#	7.1 ± 0.1a#+

Data are means ± SEM, n = 6 mussels per treatment per exposure day. Different letters within each exposure day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + indicates significant time effect compared to day 0 (stock mussels, ANOVA, $p < 0.05$).

Table 26: Na^+ concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days intermittent exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Gill	Control	1820.6 ± 88.9	1618.4 ± 43.4a	1668.4 ± 54.5a+	1393.9 ± 41.1a#+	1830.2 ± 105.2a#
	Hg alone	-	1514.3 ± 68.0a+	1655.3 ± 68.0a	1502.5 ± 44.6a+	1948.7 ± 112.6a#
	Cd alone	-	1713.2 ± 49.5a	1468.4 ± 49.5ab#+	1876.2 ± 118.2b#	2149.4 ± 60.4b#+
	Hg + Cd mixture	-	1484.0 ± 105.4a+	1351.0 ± 69.6b+	1558.0 ± 48.1a+	1720.7 ± 106.8a
Digestive gland	Control	769.9 ± 72.8	762.0 ± 45.6a	687.9 ± 77.8a	752.9 ± 92.8a	1044.7 ± 162.8a
	Hg alone	-	706.4 ± 74.1a	856.5 ± 79.3a	747.0 ± 69.9a	1360.8 ± 216.7a#+
	Cd alone	-	853.1 ± 77.0a	792.9 ± 62.9a	1167.8 ± 108.0b#+	1113.9 ± 94.1a+
	Hg + Cd mixture	-	659.1 ± 83.6a	740.5 ± 116.6a	883.1 ± 84.6a	1186.7 ±130.2a#+
Gonad	Control	690.5 ± 103.8	837.7 ± 124.3a	581.9 ± 109.8a	654.4 ± 179.5a	1497.1 ± 397.8a
	Hg alone	-	695.3 ± 147.4a	1393.1 ± 464.7a	913.7 ± 109.4a	2062.9 ± 514.2a#+
	Cd alone	-	506.9 ± 100.8a	827.1 ± 126.3a	1078.6 ± 157.6a	1561.6 ± 204.6a#+
	Hg + Cd mixture	-	543.5 ± 78.4a	826.3 ± 283.6a	679.1 ± 58.2a	1223.2 ± 216.9a

Continuation of Table 26: Na^+ concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days intermittent exposure.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Remaining Soft tissue	Control	982.3 ± 87.0	1010.7 ± 57.4a	797.9 ± 70.3a#	685.6 ± 34.2a+	1148.0 ± 98.4a#+
	Hg alone	-	942.7 ± 118.5a	1143.6 ± 78.6b	821.9 ± 119.9a	1454.1 ± 239.9a#
	Cd alone	-	901.9 ± 92.5a	908.9 ± 69.7a	934.5 ± 110.6a	1287.1 ± 117.4a#+
	Hg + Cd mixture	-	772.6 ± 75.5a	701.5 ± 58.1a+	929.4 ± 61.4a	1489.9 ± 97.4a#+
Adductor muscle	Control	776.6 ± 89.4	742.2 ± 79.6a	642.7 ± 64.2a	529.0 ± 47.4a	617.5 ± 74.5a
	Hg alone	-	780.3 ± 82.4a	772.0 ± 54.0a	660.0 ± 97.0a	1079.0 ± 202.0b
	Cd alone	-	766.6 ± 67.9a	689.0 ± 47.6a	865.5 ± 114.8a	881.3 ± 71.0ab
	Hg + Cd mixture	-	588.0 ± 79.7a	610.0 ± 56.9a	758.3 ± 57.0a	1024.0 ± 78.2b#+

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

Table 27: K^+ concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days intermittent exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$; 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Gill	Control	440.2 ± 20.0	415.9 ± 22.7a	414.1 ± 9.7a	347.7 ± 13.98a#+	388.0 ± 22.6a
	Hg alone	-	449.4 ± 26. 4a	465.1 ± 23.4b	401.8 ± 15.9b	430.8 ± 16.3a
	Cd alone	-	420.7 ± 12.9a	377.4 ± 12.7a	456.0 ± 30.0b	428.7 ± 14.5a
	Hg + Cd mixture	-	400.0 ± 22.2a	388.9 ± 15.9a	416.5 ± 6.6b	427.0 ± 15.8a
Digestive gland	Control	390.7 ± 22.3	365.5 ± 12.0a	373.0 ± 34.2a	309.6 ± 17.9a	376.9 ± 10.7a
	Hg alone	-	354.3 ± 16.4a	395.9 ± 23.6a	341.5 ± 12.9ab	439.1 ± 15.6a
	Cd alone	-	366.5 ± 19.6a	351.9 ± 10.6a	475.2 ± 27.0c#+	403.0 ± 8.1a#
	Hg + Cd mixture	-	316.0 ± 35.3a	329.2 ± 20.1a	385.3 ± 21.2b	460.2 ± 15.3a
Gonad	Control	300.1 ± 27.6	320.3 ± 42.7a	286.7 ± 40.0a	245.9 ± 44.9a	416.4 ± 45.2a
	Hg alone	-	304.3 ± 51.1a	494.0 ± 67.1a	373.2 ± 43.3ab	511.3 ± 91.1a
	Cd alone	-	301.3 ± 82.3a	334.1 ± 53.2a	483.2 ± 63.3b	396.4 ± 36.3a
	Hg + Cd mixture	-	274.4 ± 41.5a	328.0 ± 73.4a	355.3 ± 39.1ab	414.2 ± 48.5a

Continuation of Table 27: K^+ concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days intermittent exposure.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Remaining Soft tissue	Control	307.6 ± 15.6	312.0 ± 14.9a	302.6 ± 18.8a	255.6 ± 10.2a#+	319.9 ± 8.0a#
	Hg alone	-	304.4 ± 18.0a	352.6 ± 9.3b	322.4 ± 11.1b	349.8 ± 33.0a
	Cd alone	-	294.6 ± 17.1a	308. 6 ± 12.1a	341.7 ± 10.3b	332.4 ± 10.7a
	Hg + Cd mixture	-	269.1 ± 12.9a	280.9 ± 15.6a	331.0 ± 13.4b#	362.7 ± 3.7a+
Adductor muscle	Control	365.8 ± 30.9	327.1 ± 13.0a	306.2 ± 21.8a	258.8 ± 10.1a+	292.6 ± 10.7a+
	Hg alone	-	323.9 ± 16.4a	331.7 ± 23.6a	305.1 ± 12.9ac	389.6 ± 15.6b
	Cd alone	-	332.7 ± 19.6a	281.6 ± 10.6a+	370.0 ± 27.0b#	320.6 ± 8.1a
	Hg + Cd mixture	-	271.7 ± 35.3a+	281.1 ± 20.1a+	335.4 ± 21.2bc	378.7 ± 15.3b

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

Table 28: Ca^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days intermittent exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$; 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Gill	Control	71.7 ± 4.3	65.0 ± 3.2a	63.3 ± 2.3a	62.0 ± 3.1a	68.9 ± 4.2a
	Hg alone	-	64.9 ± 4.2a	70.0 ± 4.2a	59.7 ± 2.7a	80.9 ± 6.6a
	Cd alone	-	75.9 ± 2.0b	66.0 ± 2.7a	77.0 ± 4.2b	72.8 ± 2.9a
	Hg + Cd mixture	-	57.7 ± 2.8a	59.8 ± 4.1a	71.3 ± 4.6 ab	65.2 ± 3.4a
Digestive gland	Control	47.9 ± 6.9	45.1 ± 2.3ab	50.6 ± 6.7ab	48.5 ± 4.8a	53.8 ± 4.6a
	Hg alone	-	31.2 ± 3.1ac	64.4 ± 6.1a	54.1 ± 8.1a	63.6 ± 9.7a
	Cd alone	-	50.7 ± 4.0b	52.9 ± 4.0ab	88.9 ± 15.6b#+	54.7 ± 4.2a#
	Hg + Cd mixture	-	34.6 ± 3.8c	40.0 ± 2.6b	57.0 ± 6.8a	60.3 ± 6.8a
Gonad	Control	50.5 ± 3.7	35.1 ± 4.0a	35.9 ± 5.3a	37.0 ± 12.6a+	99.1 ± 23.3a#+
	Hg alone	-	47.9 ± 10.6a	148.6 ± 50.6b	80.7 ± 26.2ab	142.1 ± 40.1a
	Cd alone	-	41.5 ± 5.4a	95.7 ± 25.7b	100.1 ± 30.7b	80.1 ± 19.8a
	Hg + Cd mixture	-	36.1 ± 7.0a	30.9 ± 5.9a+	40.0 ± 3.8a	61.9 ± 7.4a#

Continuation of Table 28: Ca^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincilais* after 14 days intermittent exposure.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Remaining Soft tissue	Control	88.8 ± 13.3	60.7 ± 4.3a	68.5 ± 14.1a	55.6 ± 6.8a	73.0 ± 7.2a
	Hg alone	-	55.6 ± 6.8a	78.7 ± 7.9a	43.7 ± 5.9a#+	151.5 ± 26.7b#+
	Cd alone	-	59.9 ± 7.3a	77.6 ± 13.3a	56.0 ± 8.4a	96.2 ± 12.2a
	Hg + Cd mixture	-	75.2 ± 20.9a	51.7 ± 6.4a	66.1 ± 7.0a	161.7 ± 10.8b#+
Adductor muscle	Control	57.6 ± 13.2	36.8 ± 4.6a	34.4 ± 3.6a	40.7 ± 10.7a	35.1 ± 4.1a
	Hg alone	-	56.7 ± 13.5a	44.8 ± 6.4a	38.6 ± 8.6a	90.6 ± 21.6b
	Cd alone	-	42.3 ± 4.0a	37.5 ± 3.3a	38.5 ± 4.1a	46.8 ± 4.4a
	Hg + Cd mixture	-	26.8 ± 3.9a	42.9 ± 7.5a	45.6 ± 4.2a	61.1 ± 6.2b

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

Table 29: Mg^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days intermittent exposure to 0 (no added Cd control), 50 µg l^{-1} Hg as $HgCl_2$; 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Gill	Control	258.8 ± 8.5	248.8 ± 10.0a	235.4 ± 9.8a	217.6 ± 7.2a+	264.1 ± 13.6a#
	Hg alone	-	222.4 ± 13.1a	244.5 ± 10.6a	228.9 ± 5.0a	248.3 ± 12.0a
	Cd alone	-	245.3 ± 10.3a	222.8 ± 5.9a+	278.1 ± 18.6b#	270.6 ± 8.3a
	Hg + Cd mixture	-	223.8 ± 13.4a	210.8 ± 9.5a	237.3 ± 4.7a	230.7 ± 14.9a
Digestive gland	Control	116.6 ± 9.6	120.0 ± 6.5a	108.3 ± 9.5a	115.9 ± 12.2a	153.3 ± 21.3a
	Hg alone	-	109.6 ± 11.4a	135.5 ± 10.9a	117.7 ± 9.3a	170.1 ± 23.1a#+
	Cd alone	-	125.2 ± 10.3a	124.8 ± 8.1a	177.2 ± 14.3b#+	143.8 ± 10.0a#
	Hg + Cd mixture	-	101.6 ± 12.4a	118.2 ± 16.7a	146.1 ± 6.7ab	161.0 ± 15.5a+
Gonad	Control	97.4 ± 13.3	119.2 ± 18.9a	85.9 ± 15.4a	94.7 ± 25.5a	200.6 ± 47.0a
	Hg alone	-	96.4 ± 20.2a	196.4 ± 58.5a#	133.8 ± 15.2a	244.0 ± 56.0a+
	Cd alone	-	101.6 ± 31.6a	121.0 ± 17.5a	205.0 ± 50.4a+	186.7 ± 26.4a+
	Hg + Cd mixture	-	81.6 ± 12.1a	119.7 ± 38.1a	135.8 ± 41.3a	158.0 ± 26.4a

Continuation of Table 29: Mg^{2+} concentrations (µmol g^{-1} dw) in *M.galloprovincialis* after 14 days intermittent exposure.

Tissue	Treatments	Exposure days				
		0	2	4	8	14
Remaining Soft tissue	Control	134.9 ± 10.1	140.1 ± 6.4a	115.5 ± 8.2a	113.7 ± 10.2a	157.5 ± 13.5a#
	Hg alone	-	129.1 ± 15.1a	158.2 ± 9.8b	118.5 ± 16.2a	175.6 ± 25.8a
	Cd alone	-	128.7 ± 13.1a	131.4 ± 10.4ab	134.1 ± 14.7a	154.9 ± 12.5a
	Hg + Cd mixture	-	113.4 ± 10.1a	118.1± 13.0a	132.1 ± 7.8a	182.1 ± 10.9a#+
Adductor muscle	Control	114.9 ± 10.1	114.9 ± 11.8a	99.2 ± 7.3a	87.0 ± 6.0a	95.0 ± 10.7a
	Hg alone	-	117.6 ± 11.4a	118.2 ± 7.1a	103.0 ± 11.4ab	135.4 ± 22.5a
	Cd alone	-	115.0 ± 8.9a	108.1 ± 6.6a	130.8 ± 13.9b	113.7 ± 6.6a
	Hg + Cd mixture	-	95.7 ± 12.1a	100.3 ± 6.8a	118.5 ± 6.8b	137.1 ± 9.1a

Data are means ± SEM, n = 6/treatment on each day. Different letters within each day indicates significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + Represents significant time effect compared to time zero (day 0, stock mussels, ANOVA $p < 0.05$).

6.2.4. Effects of Hg plus Cd Mixture on TBARS

The thiobarbituric acid reactive substances, TBARS and total glutathione were measured at the beginning and end of the experiment. There were some treatment-dependent differences compared to the controls (ANOVA, $p < 0.05$, Figure 31). The Hg alone showed significant decreases in TBARS concentrations compared to the other treatments including the controls for the tissues examined (ANOVA, $p < 0.05$, Figure 31).

There was no significant difference in TBARS concentration between the Cd alone and the control for all tissues examined apart from the digestive gland and the gonad (Figure 31). TBARS concentration was significantly increased in the Cd alone compared to the Hg alone treatment for all tissue examined. There was no significant difference in TBARS concentration between the Cd alone and the Hg plus Cd mixture treatment for all tissue measured (ANOVA, $p > 0.05$, Figure 31). For the Hg plus Cd mixture, there was no significant difference in TBARS concentration between the Hg plus Cd mixture treatment compared to the controls and the Cd alone treatment. The Hg alone treatment was significantly decreased (ANOVA, $p > 0.05$, Figure 31).

6.2.5. Effects of Hg plus Cd mixture on Total Glutathione Concentration

Intermittent exposure to Hg and Cd singly, or in combination, did not result in a significant treatment effect in total glutathione concentration in all the tissues examined (ANOVA, $p > 0.05$, Figure 32). However, total glutathione was apparently depleted in the gill and digestive gland of the Hg alone treatment. For the Cd alone treatment, there was no significant effect on total glutathione concentration in all the tissues, apart from the digestive gland which showed a significant increase compared to all other treatments including the controls (ANOVA, $p < 0.05$). In the Hg plus Cd mixture treatment, total

glutathione was depleted in only the gills; there was no significant effect on total glutathione in any other tissues compared to the controls (ANOVA, $p > 005$, Figure 32).

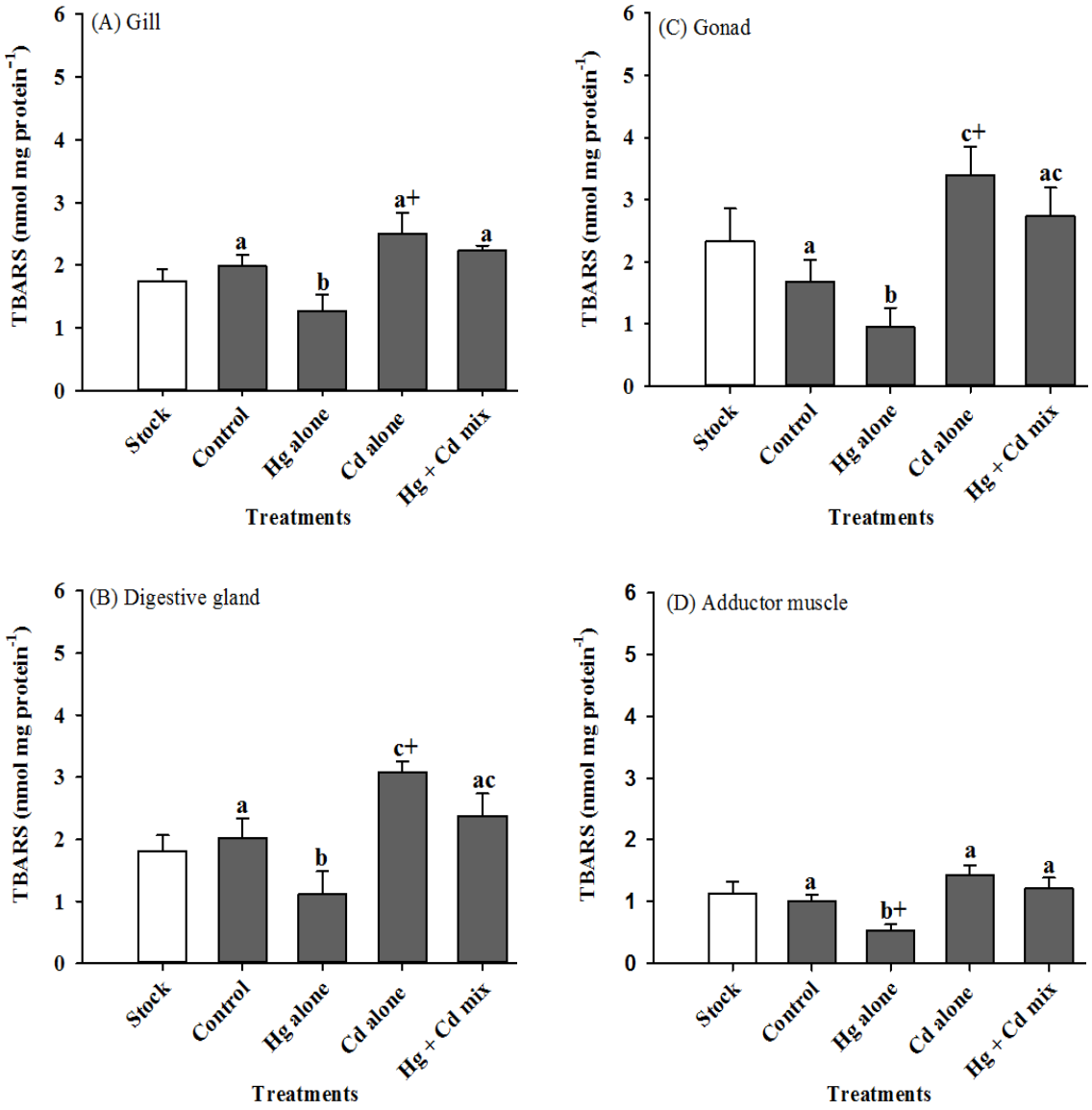


Figure 31: Thiobarbituric acid reactive substances (TBARS) concentration in the supernatant of the (A) gill, (B) digestive gland, (C) gonad, and (D) posterior adductor muscle after 14 days (grey bar) intermittent exposure to control (no added metal), 50 µg l^{-1} µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture. The white bar represents the unexposed (initial) mussels at the start of the experiment at time zero. Data are means ± SEM, nmol mg $protein^{-1}$ for n = 4-6 mussel per treatment at each exposure day. Different letters within exposure day indicates statistically significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, stock mussel; ANOVA, $p < 0.05$).

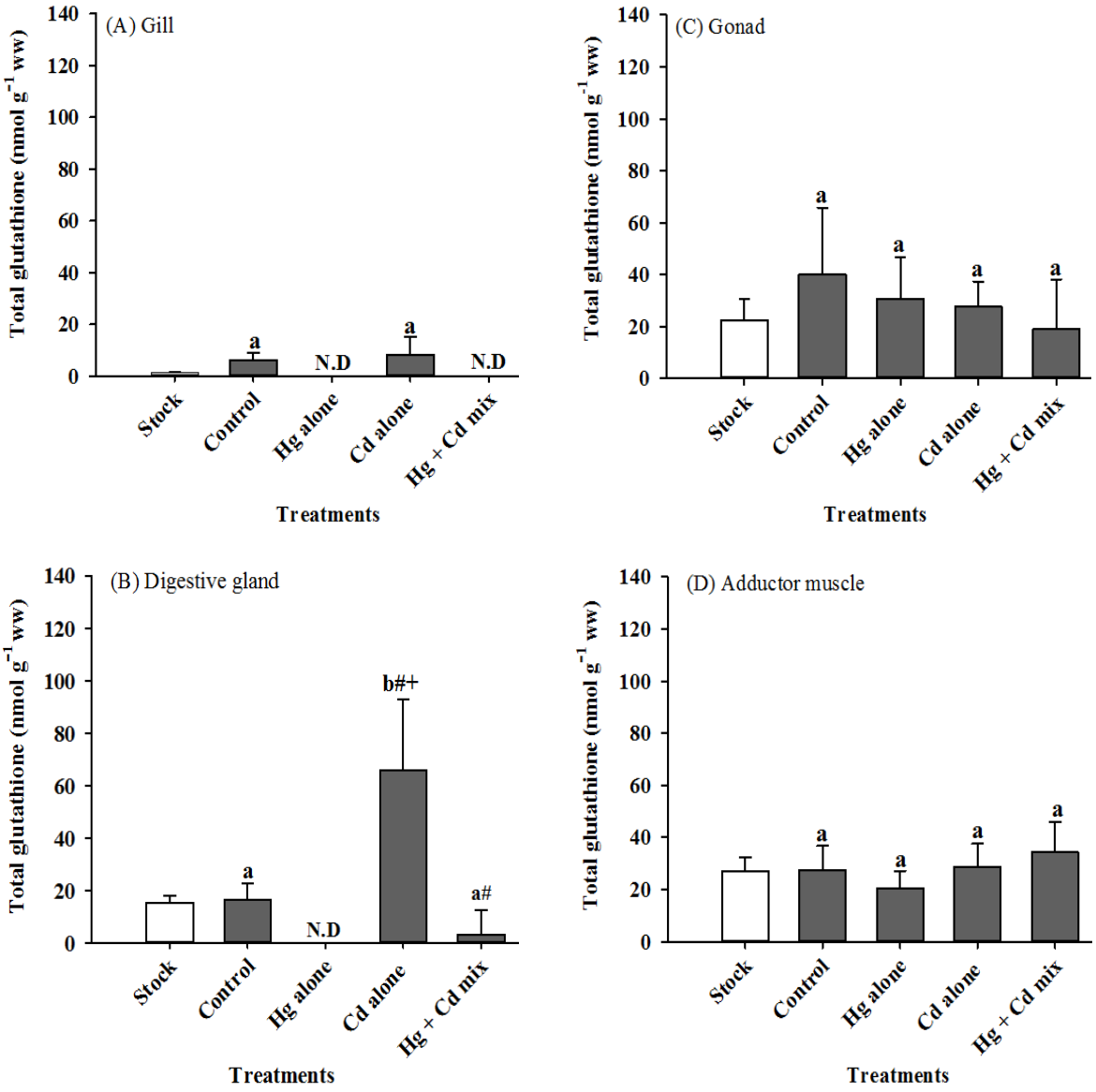


Figure 32: Total glutathione concentration in the supernatant of the (A) gill, (B) digestive gland, (C) gonad, and (D) posterior adductor muscle after 14 days (grey bar) intermittent exposure to control (no added metal), 50 µg l^{-1} µg l^{-1} Hg as $HgCl_2$, 50 µg l^{-1} Cd as $CdCl_2$ and 50 µg l^{-1} each for the Hg plus Cd mixture. The white bar represents the unexposed (initial) mussels at the start of the experiment at time zero. Data are means ± SEM, nmol g^{-1} ww for n = 4-6 mussel per treatment at each exposure day. Different letters within exposure day indicates statistically significant treatment effect (ANOVA, $p < 0.05$). # indicates significant time effect within treatment compared to the previous exposure day (ANOVA, $p < 0.05$). + represents a significant time effect compared to day zero (day 0, stock mussel; ANOVA, $p < 0.05$). Note N.D represents not detected.

6.2.6. Histological Alterations during Continuous Exposure to Hg and Cd Mixture

At the end of the 14 day intermittent exposure to Hg and Cd singly or in combination, there was no observed damage to the gill filaments of the unexposed control animals. The control mussels showed the normal architecture of the frontal face comprising of the frontal cilia, lateral cilia and the laterofrontal cilia. There was no observed hyperplasia, haemocyte infiltration or any other damage in all 6 gill samples examined (Figure 33A).

There were observed treatment-dependent effects in the metal treatment compared to the unexposed control (Figure 33B). All mussels (6/6) in the Hg alone treatment showed hyperplasia in the tips of the gill lamella and dequamation of epithelial cells. There was no observed pathology in the Cd alone treatment apart from the hyperplasia in the tips of the gill lamella in all six mussels examined (Figure 33C). All mussels in the Hg plus Cd mixture showed hyperplasia in the tip of the gill lamella. Two out of six mussels showed desquamation of epithelial cells. One out of six mussels showed oedema characterised by swelling of the gill lamella.

Like the gill, there was no observed pathology in the digestive gland of the control animals. The normal architecture of the digestive gland tubules (oval or round) was maintained. There was no observed haemocyte infiltration in the connective tissues, neither was there any other pathology (Figure 33D). There was no significant treatment-dependent difference in the epithelial cell height (ANOVA, $p = 0.50$). Values (means ± SEM, $n = 6$, µm) were 40.7 ± 2.3, 33.9 ± 2.0, 33.6 ± 1.7 and 33.6 ± 1.0 for control, Hg alone, Cd alone and Hg plus Cd mixture respectively.

Four out of six mussels in the Hg alone treatment showed haemocyte infiltration in the connective tissues of the digestive gland. Degeneration (breakdown of the tubule due to high level of haemocyte infiltration) of the tubule architecture was observed (1/6

animals). Two out of six mussels showed desquamation (peeling) of the digestive cells (Figure 33F). For the Cd alone treatment, 2/6 mussels showed slight haemocyte infiltration in the digestive tubules (Figure 33G). All mussels (6/6) in the Hg plus Cd mixtures showed desquamation of the digestive cells. Four out of six showed inflammation characterised by haemocyte infiltration. Degeneration of the tubule architecture was observed in 2/6 mussels.

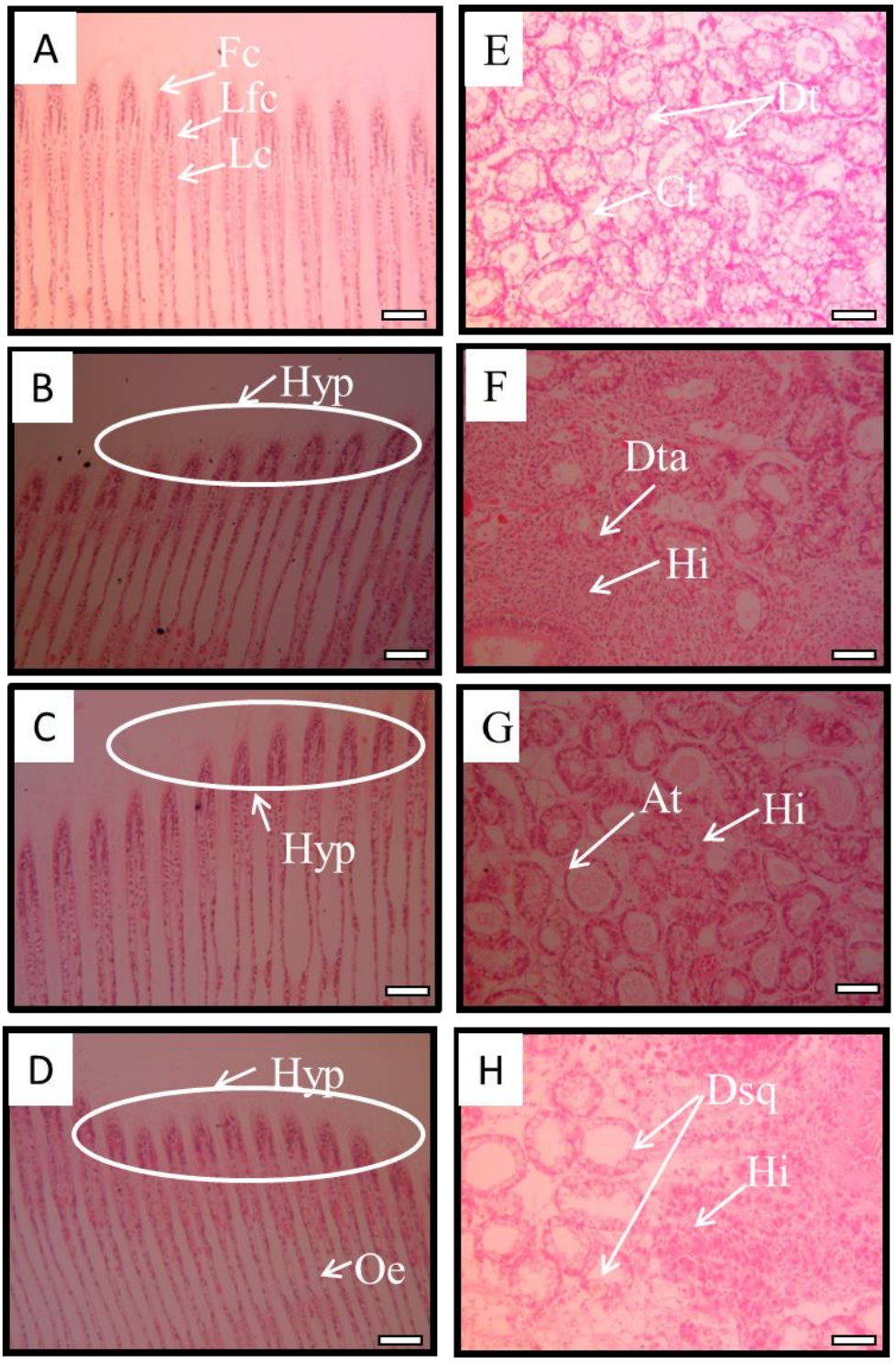


Figure 33: Histology of the gill (A-D) and digestive gland (E-H) of *M. galloprovincialis* after 14 days intermittent exposure to control (no added metal, A, E), 50 µg l^{-1} Hg as $HgCl_2$ (B, F), 50 µg l^{-1} Cd as $CdCl_2$ (C, G) or 50 µg l^{-1} Hg plus Cd mixture (D, H). Slides were stained with Mayer's Haemotoxylin and eosin. Circle represents gill filaments with hyperplasia (Hyp) on the tips. At = atrophy; Ct = connective tissues; Dt = digestive tubules; Dta = degeneration of tubule architecture; Fc = frontal cilia; Lfc = laterofrontal cilia; Lc = lateral cilia;Hyp = Hyperplasia; Oe = oedema; Scale bar: 50 µm.

6.3. Discussion

6.3.1. Water Quality during Aqueous Exposure to a Mixture of Hg and Cd

The nominal concentration of 50 µg l^{-1} per metal in the seawater was confirmed by the measured concentrations in the tanks (Figure 27). There were no differences in the seawater metal concentration between the single and the mixture treatment for both Hg and Cd. Daily water quality parameters measured were not different in all tanks including the control. The result in the present study suggests that the mussels were not stressed in reference to the parameters measured.

6.3.2. Tissue Accumulation and Metals Interaction during Exposure to a Mixture of Hg plus Cd

Intermittent exposure to Hg and Cd singly and in mixture did not result in treatment-dependent effect in the tissues examined except for the haemolymph (Hg, Figure 28) and gill (Cd, Figure 29). The increase has previously been reported (section 5.3.2, this thesis) to be as a result of Hg positively influencing the uptake of Cd. Statistically, there was no difference between the sums of the individual metals compared to the actual data of the mixture (Figure 30), suggesting additive (no interaction) effect. The no interaction in accumulation suggests that the pathway of uptake of Hg and Cd were dissimilar. This idea has been previously reported (section 5.3.2, this thesis).

6.3.3. Effects of Hg plus Cd Mixture on the Haemolymph Chemistry and Ion Regulation

The exposure of mussels to Hg or Cd singly or in mixtures in the present study, had no effect on the total haemocyte counts and cell-free haemolymph glucose. Neither was there any effect on osmotic pressure, Na^+ and K^+ concentrations in the cell-free haemolymph. However, the haemolymph protein in the Hg alone treatment was affected. There are at least two explanations to the haemolymph protein viz (a) leakage of protein from the tissues (b) inflammatory response. The first option does not seem to be the case since there was no change in other extracellular fluid for such as the the Na^+, K^+ concentration (Table 24). The increase in the haemolymph protein interpreted as the immune system responding to inflammation from the exposure. This idea is evident in another section (section 6.2.6, histology) of this thesis, where Hg-mediated inflammation was observed in the gill and digestive gland of mussels exposed to either Hg alone or the Hg plus Cd mixture treatment.

Also, the major electrolytes (Na^+, K^+, Ca^{2+} and Mg^{2+}) examined in the tissues (gill, digestive gland, remaining soft tissue, gonad and adductor muscle) showed some treatment-dependent effect at the end of the experiment. For example the gill showed increases in Na^+ concentration in the Cd alone treatment compared to the other treatments including the controls. This reason for this increase is unclear.

6.3.4. Oxidative Stress and Organ Pathology during Exposure to a Mixture of Hg and Cd

Mercury and cadmium are known causes of lipid peroxidation and oxidative stress (Garron et al., 2005; Liu et al., 2009). However, the in the present study, exposing mussel intermittently to Hg and Cd singly or in mixture did not cause any lipid

peroxidation as measured by the TBARS (Figure 31). Neither was there any effect on total glutathione concentration apart from the Cd alone treatment in the digestive gland (Figure 32). This is evident especially in the gonad and the adductor muscle where there were no significant difference between the metal treatment groups and the unexposed controls for total glutathione concentration. The concept of decreased total glutathione concentration and increased TBARS concentration as an indication of oxidative stress has been established (Halliwell and Gutteridge, 1999; Filho et al., 2001). However, the total glutathione concentration in the digestive gland of the Cd alone treatment showed a significant increase compared to the unexposed control and the other treatment. The result is consistent with the idea that glutathione is a non-enzymatic antioxidant that protects the cells against reactive oxygen species (ROS) by scavenging them (Santovito et al., 2005). Oxidative stress occurs when the rate of the ROS production exceeds the rate of its deposition by the antioxidant, leading to an increase in oxidative damage to different cellular targets (Almeida et al., 2005). The results suggest that there was no overall oxidative stress on mussels by the exposure.

The histological examination showed increased injury in the gill and digestive gland compared to the Hg alone or the Cd alone treatment (Figure 33), suggesting synergism. Hyperplasia as observed in all the metal (s) treatment groups suggests that the mussels were reacting to the exposure. Inflammation characterised by the infiltration of the haemocyte as observed might be part of the protective response of the immunoactive haemocytes to the exposure. The idea of haemocytes migrating in response to metal toxicity has been reported previously (Marigómez et al., 1990; Amachree et al., 2013). Desquamation and oedema (Hg plus Cd mixture only) as observed in the Hg alone and Hg plus Cd mixture indicates that cells were target for Hg toxicity. As aforementhioned, epithelial cell damage is believed to be as a result of

increased lipid peroxidation (Gstraunthaler et al., 1983; Lund et al., 1993). However, this was not the case in the present study, TBARS concentration was not increased in any of the tissue (Figure 31).

6.3.4: Conclusions

To the best of our knowledge, this is the first report on the accumulation and sub-lethal effect after aqueous exposure to Hg and Cd single or in mixture to *M. galloprovincialis* using an intermittent profile. The result of the study has demonstrated that exposing mussel intermittently to a combination of Hg plus Cd did not produce any toxic response. Nor was there any effect of the individual metal exposures.

Chapter 7 *:*

General Discussion

7.0. General Discussion

The metals (Hg and Cd) used in the present study are listed among the priority substances by the Water Framework Directive (WFD), Directive 2000/60/EC. Both chemicals occur together in nature (Zn and Cu ores), and are often times released together into water bodies via anthropogenic sources (e.g., mining, industrial effluents and municipal solid waste). Hg and Cd are bioaccumulative, non-essential metals thus do not have any biological role in the organism. The nominal concentration (50 µg l^{-1}) of Hg or Cd used here is a known sub-lethal concentration with no mortality and has been previously reported on *M. edulis* in our laboratory (Sheir et al., 2010; Sheir and Handy, 2010).

The work in the Chapters 3, 4, 5 and 6 enables comparison between continuous and intermittent exposure. However, it is important to bear in mind some caveats. Firstly, the experiments were performed at different times of the year (Table 30); chapters 3 and 4 were within the summer months while chapters 5 and 6 were performed during the winter months. Secondly, for logistic reasons the continuous and intermittent exposures for the Hg plus Cd mixture were done in separate experiments, chapters 5 and 6 respectively. Given these caveats, it is important to note that all the exposures and endpoints measured in this thesis were performed exactly the same way to enable comparison.

Table 30: Thesis experimental chapters, number of mussels and date of collection from Port Quin.

Thesis Chapter	No of mussels	Date of collection from Port Quin
3	198	September, 2010
4	198	August, 2011
5	132	November, 2011
6	132	October, 2012

7.1. Accumulation of Hg or Cd: Comparison during Intermittent and Continuous Exposure

In the present study, metals (Hg, Cd) were exposed via the aqueous route; uptake was mainly through the gills, then redistribution into the other organs. The target organs were the same for Hg and Cd exposed singly. Trace metals (Hg and Cd) analysis was carried out in the tissues (gills, digestive gland, gonad, adductor muscle, remaining soft tissue and haemolymph) of *M. galloprovincialis*. Hg was highly accumulated in the internal organs with the gill showing the highest (≤ 2 fold than the digestive gland) and the adductor muscle accumulating the less total Hg (Figures 15, 21 and 28). The result suggested a slower efflux of the Hg across the gill to the haemolymph resulting in high levels of Hg accumulation in the gill. This is expected; as the gills serve as both uptake site and reservoir for Hg (Roesijadi et al., 1981). The same situation was shown in the Hg exposed gills throughout this thesis and is in agreement with previous findings after continuous exposure to Hg (Sheir et al., 2010; Roesijadi et al., 1981). The accumulation of Hg during continuous exposure was the time-dependent linear accumulation pattern for all tissues examined. Likewise, all tissues in the intermittent regimes showed a time-dependent increasing trend apart from the gill. At the end of the experiment, the gill and the adductor muscle did not show significant difference in Hg accumulation between the continuous and intermittent exposure. The gill showed a statistically significant step-wise increase, which was demonstrated by the alternating uptake and clearance pattern (Figure 15). However, this trend was not evident in the Hg + Cd mixture experiment during intermittent exposure (Figure 28). The reason for this was due to the sample collection times in the different experiments, though the exposure procedures were exactly the same (2 days exposure: 2

days placed in clean water). For Figure 15 samples were collected on days 0, 2, 4, 6, 8, 10, 12 and 14, while those of Figure 28 were collected on days 0, 2, 4, 8 and 14 showing only day 4 and 8 as the intermittent period. Hg concentration at the end of the experiments were within the same range, values were (means ± SEM, n = 6), 1358.8 ± 176.3 (Figure 15), 1306 ± 235.9 and 1202.2 ± 192.2 (Figure 28, Hg alone and Hg + Cd mixtures respectively), indicating the experiments were properly executed. Like the Hg, Cd was also accumulated in all the tissues examined (Figure 8). Mussels tend to accumulate Cd slightly more in the digestive gland compare to the other tissues. Overall, by the end of the experiment, Cd accumulation was significantly higher in the continuous compared to the intermittent for gill, digestive gland, the remaining soft tissue the haemolymph but not the gonad and adductor muscle (Figure 8).

7.2. Accumulation of Hg and Cd Mixtures: Comparison during Intermittent and Continuous Exposure

For the Hg plus Cd mixture experiment, mussels were exposed to metals singly and in mixtures in the same experiment to enable easy comparison of the effects in the continuous (Chapter 5) as well as the intermittent exposure (Chapter 6). Target organs were the same in both the continuous and intermittent exposure. There were no differences in Hg concentration between the Hg alone and the Hg plus Cd mixture (Chapters 5-6). Neither were there any difference between the Cd concentrations of the Cd alone and the Hg plus Cd mixture treatment apart from the gill (Chapters 5-6). There was also no evidence of competitive metal accumulation, thus no interaction between the Hg and Cd during accumulation (Chapter 5 and 6, Hg plus Cd mixture). Overall, the result shows that Hg was accumulated more than Cd especially in the gill.

Hg and Cd have similar chemistry; they are both class 'b' metals and have affinity for the same type of biotic ligands (cell types rich in sulphur and nitrogen ligands). However, Hg is said to be easily taken up from solution while Cd is taken up largely with particulate matters, resulting in low and high toxicity respectively (Akberali and Trueman, 1985). This might be part of the reason why Hg is accumulated more in the gill and Cd in the digestive gland in the present study.

7.3. Biological Effects of Hg or Cd: Comparison during Intermittent and Continuous Exposure

Trace metals are known to exert their effects on organism either through direct action on the tissues or from subtle alterations in the homeostatic mechanisms such as the immune system (Auffret and Oubella, 1997). In the present study, the endpoints measured reflect the main physiological processes affected by the two metals (Hg, Cd) in areas of osmoregulation (tissue and plasma electrolytes, osmotic pressure); oxidative stress parameters (lysosomal membrane damage via neutral red retention, total glutathione, thiobarbituric acid reactive substances or TBARS); as well as organ pathologies and haematology.

There was no overall treatment effect on the plasma glucose during continuous or intermittent exposure to Cd or Hg (Chapters 3 and 4 respectively). According to Lagadic et al. (1994), invertebrate haemolymph glucose is insensitive to contamination. Neither was there any effect on neutral red retention regardless of the exposure regime (Chapters 3 and 4). Total haemocyte count was not affected in the entire experiment except for the Hg alone treatment (Chapter 4). In the Hg alone treatment, the total haemocyte count was significantly increased (continuous) and decreased (intermittent).

Response of the haemocytes is general to chemical exposure (Coles et al., 1995). This response is evident in the histological examination of tissue exposed to Hg where tissues showed inflammatory responses characterised by haemocyte infiltration (continuous and intermittent exposure) and granulocytomas (intermittent exposure) (Chapter 4, histology section). Together this suggests that the immunological ability of the haemocytes were still intact regardless of the exposure regime. There was no overall effect on the osmoregulatory function of mussels caused by the exposure to Hg, Cd alone or mixtures during continuous and intermittent exposure. The osmotic pressure, Na^+ and K^+ concentration of the cell-free haemolymph was not affected by the exposure but remained in the normal range for mussels. However, they were some transient changes in the tissue electrolytes (Na^+, K^+, Ca^{2+} and Mg^{2+}). Overall, there was no adverse effect in the electrolytes; mussels were placed in normal seawater throughout the experiment. *M.galloprovincialis* is an osmoconformer. In extreme situation, the valve closing mechanism allows time for acclimatization to the external medium. Electrolyte concentrations in the mussel have been argued to be similar to those of the external medium as long as the shells are open (Shumway 1977).

Hg and Cd are known causes of lipid peroxidation and subsequent oxidative stress (Garron et al., 2005; Liu et al., 2009). Exposing mussels to Cd alone during continuous and intermittent exposure did not show any major effect on tissue TBARS concentrations. However, TBARS concentration was increased by Hg exposure in the continuous (Chapter 4 and 5) but not intermittent exposure. This suggests that continuous exposure to Hg causes an oxidative challenge that is not seen in the intermittent event. There was no effect on total glutathione at the end of the experiment throughout the thesis, except for the apparently depleted in gill of the Hg exposed

groups (Chapter 4). This apparent depletion has been attributed to Hg interfering with the assay in use in this thesis (see Chapter 4 for details).

Histological examination as useful tools uses to determine specific alteration in the tissue in response to chemical exposure. Detailed quantification was made on the histological responses in the present thesis (Chapters 3 and 4). Tissues (gill and digestive gland) exposed to mercury showed more severe alterations compared to those exposed to Cd alone experiment (Chapters 3 and 4). Only hyperplasia was seen in the gill of the Cd exposed tissues for both continuous and intermittent exposure. Continuous exposure to Hg resulted also in hyperplasia alongside oedema (swelling of the lamella) (Chapter 4). The gill of mussels serve dual purposes (respiratory and feeding), alteration in the structure may impair these functions, which may also lead to reduced growth and mortality. To clarify these supposed impaired functions, further investigation on the clearance and oxygen consumption rates should be incorporated in future experiments. In the digestive gland, there was no difference in effect between the continuous and intermittent exposure, Cd exposure resulted in haemocyte infiltration and desquamation of the digestive cells in both exposure regimes (Chapter 3).

7.4. Biological Effects of Hg and Cd Mixture: Comparison during Intermittent and Continuous Exposure

As with the single metals, exposing mussels to a mixture of Hg and Cd did not have overall treatment effect on the plasma glucose during continuous or intermittent exposure (Chapters 5-6). No effect was observed on neutral red retention or total haemocyte counts regardless of the exposure regime (Chapters 5 and 6). The osmotic pressure, Na^+ and K^+ concentration of the cell-free haemolymph and tissue electrolytes

(Na^+, K^+, Ca^{2+} and Mg^{2+}) were not affected as well by the Hg plus Cd mixture both remained in the normal range for mussel.

Hg and Cd are known causes of lipid peroxidation, exposing mussels to Hg plus Cd mixture during continuous and intermittent exposure did not show any major effect on tissues TBARS concentrations. However, the Hg alone control of the same experiment showed increased in TBARS concentration (Chapter 5). This suggests that continuous exposure to Hg causes an oxidative challenge. However, this effect was not seen when mussels were exposed alone to Hg (Chapter 4), suggesting that Cd has a buffering effect on Hg toxicity. No explanation to this comes to mind. There was no effect on total glutathione at the end of the experiment throughout the thesis, except for the apparent depletion in gill of the Hg exposed groups (Chapter 5 and 6). This apparent depletion has been attributed to Hg interfering with the assay in use in this thesis (see Chapter 4 for details).

Histological examination of the gill and digestive gland was also performed for the mixtures (Chapters 5 and 6). Hg exposure resulted also in hyperplasia alongside oedema (swelling of the lamella) in the continuous but only hyperplasia (Chapter 5 and 6) and desquamation of the gill epithelial cells (Chaper 6). In the digestive gland histology, there was no difference in pathology between the continuous and intermittent exposure for the Hg plus Cd treatment (5 and 6). The pathology in the mixtures were similar to that of Hg alone treatment, in the same experiment, suggesting Hg influence in the tissue pathology.

7.5. Is the Bioaccumulation Response During Intermittent Different From the Continuous Counterparts?

The major research question of this present study was whether metal accumulation and responses of *M. galloprovincialis* exposed to metals during intermittent and continuous exposure are different. Of course, there were differences. Nevertheless, the differences were not those that were expected. It is expected that accumulation of metals (Hg or Cd) in the intermittent will be half as much as those in the continuous exposure since mussels in the intermittent regime were exposed half as long as those in the continuous regime. The idea was that since dose equals concentration of the contaminant multiplied by the time of the exposure (i.e. dose = concentration x time): half the concentration of metal (s) and half the time of exposure should result in half the dose in the intermittent compared to the continuous exposure. It is interesting to know that accumulation of the metals were more than half in some tissues (Chapters 3-6). For example, Accumulation in the intermittent was equal (as in the case of gill Hg concentrations) or more than half of the continuous exposure (Chapters 4, 5 and 6).

Also, the physiological responses were mostly similar in both exposure regimes. However, Hg tends to cause more disturbances in the intermittent than the continuous exposure. In general, bioaccumulation responses may be equal, less or more than in the intermittent compared to the continuous.

It is important to note that the factors influencing metal uptake (e.g., size, temperature, salinity, physiological state of the animals) were taken into consideration. In the present study the size (40-60mm) of mussels, temperature (15 ± 1 ºC) of the media was controlled and mussels were maintained the same way all through the experiments. There was no difference in the salinity, pH, DO and ammonia

concentration in all of the exposure periods. Hence, these factors were not considered to be affecting the comparison in the present study.

7.6. Limitation and Future work

This is the first report that uses in addition to accumulation, responses at different levels of biological organisation (i.e., biochemical, cellular and physiological levels) to compare the sub-lethal effects of Hg and Cd exposed either alone or in mixture to the mussel, *M. galloprovincialis* during continuous and intermittent exposure. The study was able to answer the research questions, ‘’the bioaccumulation responses of intermittent exposure will be the different from the continuous exposure in an equal peak concentration’’. However, there are a few limitations in the approach used in the present study with regards to risk assessment and environmental protection, they include:

- ***The sensitivity of mussels compared to other marine organisms and life stages***

The aim of risk assessment is to provide basic information on the amount of contaminants introduced into the environment and develop regulations that are capable of reducing the adverse effects of the contaminants on the ecosystems (Vighi and Villa, 2013). Choosing the right reference organism in terms of their exposure-to-dose and dose-to-effect is pertinent in risk assessment (Brèchignac and Doi 2009). Several organisms (microalgae, daphnia and zebrafish) have been explored and documented as reference organism for environmental risk assessment (ERA). However, in contrast to the vast number and variety of species occupying various trophic levels in real

ecosystems, the present study uses *M. galloprovincialis*, a sentinel species. Tissue metals accumulation in the present study was relatively high without evident of adverse biological effects (Chapter 3-6). There is the concern for using such hardy species in ecotoxicological investigations, hence its logical to use species with short life histories and more sensitive responses (e.g., microalgae, daphnia and zebrafish) to yield more accurate and ecologically relevant results (Wilson, 1994).

It is also important to bear in mind that a single species as used in the present study may not fully represent the impact of contaminants on the biota. Knowing that sediment serves as sink to contaminant (Mason and Lawrence, 1999), different organisms occupy different trophy levels and organismal responses to contaminants are depended on both the biotic and abiotic (e.g., temperature, salinity, pH, DOM especially for metals) factors (Wang et al, 2010; Bidwell, J. R., Gorrie, J. R., 2006; Bervoets and Blust 2000). Also a single life stage (e.g larvae, juvenile or adult) cannot protect the population. Reports have shown that the early life stages of bivalve molluscs are more sensitive to some contaminants than their adult equivalent or some commonly tested aquatic organisms (Wang et al., 2010; March et al., 2007; Damien et al., 2006; Williams and Hall, 1999). It is essential to measure a suite of ecologically representative organisms occupying different ecological niches across the levels of biological organisation to assess the impact of the contaminant as well as the ecological integrity (Brèchignac and Doi, 2009; Clements, 2000).

- ***Predication of toxic impact on population by biomarkers?***

One question that comes in mind during ecotoxicological studies in the laboratory is whether the biomarkers are able to predict the toxic impact on the

population. According to Vighi and Villa (2013), Cheung et al. (2006) and Power and McCarty, (1997) extrapolation of the results from laboratory-based studies may not predict the actual consequences on the biota or the structure and function of the ecosystem. Ecotoxicology covers a wide range of responses from contaminants at the different organisational levels (Figure 34): It aims at protecting the ecosystem by using responses from ecologically representative organisms covering different trophic levels to detect ecosystem damages (Vighi and Villa, 2013; Moore et al., 2004). Protection becomes more challenging at higher levels of organisation (population, communities and ecosystems) where biological endpoints are more complex and difficult to measure (Figure 34, Vighi and Villa, 2013; Moore et al., 2004).

It has been reported that the effects of contaminants on aquatic organisms may occur or manifested at one or all levels of the biological organisation (i.e from the molecule up to the ecosystem, Figure 34). And no single level is representative of the effects from contaminants; therefore studies should cut across multiple levels of biological organisation simultaneously (Clements, 2000). The rational for using the multiple biomarker approach is that, indicators at the different levels will provide different types of information needed for ERA. Although, ecotoxicology interest on the effects on population and communities are more ecologically relevant; it is necessary to measure responses (e.g., molecular, cellular, physiological and biochemical responses) at lower levels. These responses occur rapidly, specific and may serve as early warning to toxicological effects on population as they may be directly linked to exposure (i.e., a cause and effect relationship between stressors and response, Figure 34, Clements, 2000). The one solution to this ecotoxicological problem is to develop a mechanic link that will establish a cause to effect relationship of ecological relevance at the different level of organisation (Clements, 2000).

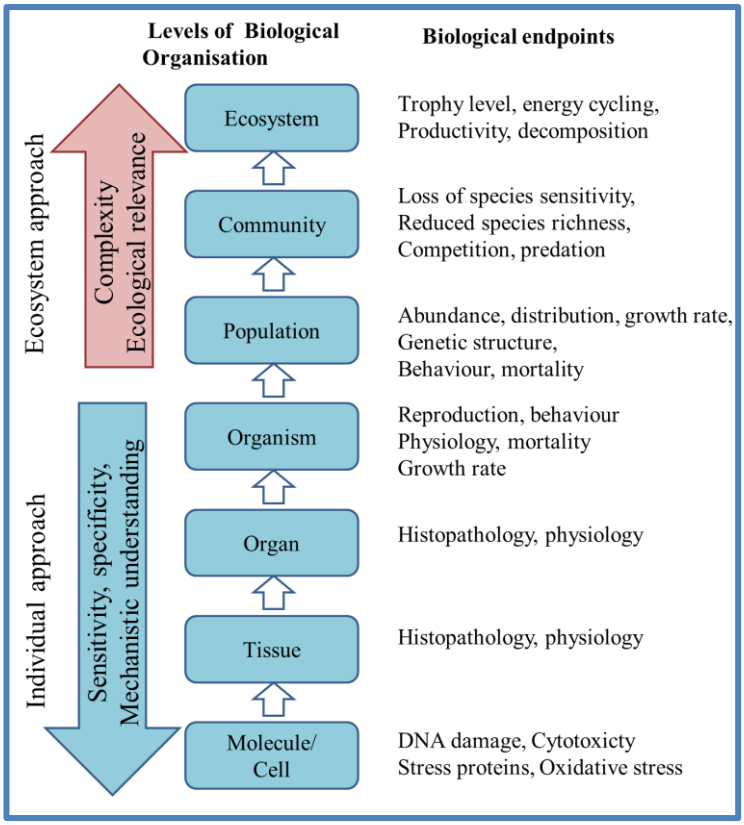


Figure 34: Assessment of biological responses across the levels of biological organisation.

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